

# Identification and role in virulence of putative iron acquisition genes from *Corynebacterium pseudotuberculosis*

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## Abstract

Four genes, *fagA*, *B*, *C* and *D*, encoding products with 32–47% identity to proteins involved in bacterial iron uptake systems, were identified immediately downstream of the *Corynebacterium pseudotuberculosis* phospholipase D gene.  $\beta$ -Galactosidase assays on a *C. pseudotuberculosis* strain carrying a *fagA-lacZ* fusion indicated that the putative *fagABC* operon was poorly expressed in iron-rich media. However, similar experiments in iron-limited media resulted in an approximately three-fold increase in  $\beta$ -galactosidase activity, suggesting that this operon is regulated by iron in vitro. Although no defect in iron utilization could be determined for a *C. pseudotuberculosis fagB(C)* mutant in vitro, this mutant showed reduced virulence compared to wild-type in a goat model of caseous lymphadenitis. Thus, expression of the *fag* genes in the host appears to contribute to virulence. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Caseous lymphadenitis; Iron transport; Intracellular survival; Virulence

## 1. Introduction

*Corynebacterium pseudotuberculosis* is the causative agent of caseous lymphadenitis (CL) in goats and sheep [1]. Infections are generally confined to the lymphatic system, with abscessation and granuloma formation in the lymph nodes, but may become systemic, spreading to the lungs and other organs [1]. This organism produces a toxic phospholipase D, which allows dissemination and persistence of *C. pseudotuberculosis* in the lymphatic system [2]. However, *C. pseudotuberculosis* is also capable of surviving and proliferating within macrophages [3,4], which may be its method of translocation through the lymphatic system. Therefore, like other intracellular bacterial pathogens, *C. pseudotuberculosis* must be able to acquire iron within the low iron environment of the host cell.

In this paper we report the identification of four *C. pseudotuberculosis* genes, *fagA*, *B*, *C* and *D* encoding proteins with similarity to iron ABC transporters from other bacteria. We demonstrate the regulation of the pu-

tative *fagABC* operon by iron and the involvement of these genes in the ability of *C. pseudotuberculosis* to persist in a goat infection model.

## 2. Materials and methods

### 2.1. Bacterial strains and media

All *Escherichia coli* strains used in this study were derivatives of *E. coli* strain DH5 $\alpha$  (Gibco-BRL) and were grown on either Luria–Bertani (LB) agar or in LB broth at 37°C. 100  $\mu$ g ml<sup>-1</sup> ampicillin or 30  $\mu$ g ml<sup>-1</sup> chloramphenicol (Cm) were added, as appropriate. All *C. pseudotuberculosis* strains used in this study were derivatives of the caprine isolate, Whetten 1 [5] and were grown on brain heart infusion (BHI) agar supplemented with 5% bovine blood, at 37°C. Liquid cultures of *C. pseudotuberculosis* were grown in BHI broth supplemented with 5% fetal calf serum at 37°C with shaking. For  $\beta$ -galactosidase ( $\beta$ -gal) assays of transcription fusions, *C. pseudotuberculosis* strains were grown in BHI broth supplemented with 0.1% Tween 80, with (iron-limited) or without (iron-rich) 20  $\mu$ g ml<sup>-1</sup> 2,2'-dipyridyl. *C. pseudotuberculosis* cultures were supplemented with 5  $\mu$ g ml<sup>-1</sup> Cm, where appropriate.

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## 2.2. Utilization of iron sources by *C. pseudotuberculosis*

Approximately  $10^7$  washed *C. pseudotuberculosis* cells were plated onto the surface of BHI agar medium containing 50  $\mu\text{M}$  ethylenediamine-di(*o*-hydroxy-phenylacetic acid) (EDDHA). This concentration of EDDHA completely inhibited growth of *C. pseudotuberculosis* strains unless an exogenous iron source was added. Wells, 5 mm in diameter, were made in the agar and 10  $\mu\text{l}$  of the following iron sources were placed in the wells; 1 mM  $\text{FeCl}_3$ , 1 mM  $\text{FeSO}_4$ , 1 mM hemin, 10  $\mu\text{M}$  hemoglobin, 30  $\mu\text{M}$  transferrin and 1 mg  $\text{ml}^{-1}$  ferrichrome. Plates were incubated at 37°C for 48 h and assessed for bacterial growth.

## 2.3. DNA techniques

*E. coli* transformation and plasmid extraction, DNA restriction, ligation, agarose gel electrophoresis and Southern hybridization were performed as described [6]. PCR DNA amplification was performed using *Taq* DNA polymerase (Promega) for 35 cycles consisting of 1 min at 94°C (DNA denaturation), 1 min at 55°C (primer annealing) and 1 min  $\text{kb}^{-1}$  at 72°C (DNA synthesis).

## 2.4. Nucleotide sequencing and computer analysis

The nucleotide sequence of a 4256-bp region downstream of the *C. pseudotuberculosis* Whetten 1 *pld* gene was determined from appropriate subclones of the *pld*-encoding cosmid pCpO1 [5], including pCpO48 [5] and pCpO51s [2]. Sequencing was performed on both strands, crossing all restriction sites, on a 377A DNA sequencer (Applied Biosystems Inc.) at the Automated DNA Sequencing Service at the University of Arizona. Nucleotide sequence data were compiled using the Sequencher™ 3.1 program (GeneCodes). Database searches were performed using the BlastN, BlastX and BlastP algorithms [7] and the presence of conserved patterns determined using InterProScan [8]. Similarity was determined from optimized sequence alignments using the CLUSTAL W program [9]. Predictions of signal sequences, lipoproteins and transmembrane domains were performed using the PSORT program [10].

The nucleotide sequence data reported in the paper have been deposited in the GenBank nucleotide sequence database under the accession number AF401634.

## 2.5. *lacZ* transcriptional fusions and $\beta$ -galactosidase assays

A 509-bp PCR product encompassing the *fagA-fagD* intergenic region was amplified using the primers 5'-agccttgcgggatccatgacgctgg-3' and 5'-atccaaaggatccacacgtttagg-3'. This PCR product was inserted into pJF751 [11], utilizing the *Bam*HI sites in the primers, such that the first

51 codons of *fagA* were fused in frame with the *lacZ* open reading frame (ORF) of pJF751 (pJGS266). A 1255-bp PCR product containing the *pld* promoter was amplified from pCpO48 with the universal M13 primer 5'-acgttg-taaaacgacggccagt-3' and a second primer 5'-ataaaacaactg-gatccctcat-3', digested with *Bam*HI and inserted into the *Bam*HI site of pJF751 such that the first three codons of *pld* fused with the *lacZ* ORF, to create pJGS188. Since the pJF751 replicon does not function in *C. pseudotuberculosis*, pJGS188 and pJGS266 were each introduced into Whetten 1 by electroporation [12], selecting for Cm resistance, which would indicate incorporation of the *lacZ* fusion into the chromosome via a Campbell recombination event. The construction of *pld-lacZ* (JGS343) and *fagA-lacZ* (JGS378) fusion strains was confirmed by PCR and Southern hybridization (data not shown). Cultures of JGS343 and JGS378 were grown to an  $\text{OD}_{600} = 1$  in iron-rich or iron-limited media. Cells were disrupted by two passages through a French pressure cell (Aminco) at 138 MPa.  $\beta$ -Gal assays were performed on cell lysates as described by Miller et al. [13]. Assays were performed in duplicate on three independent cultures grown in iron-rich or iron-limited media. Protein concentrations were determined using a Bradford assay (Bio-Rad).  $\beta$ -Gal activities were calculated in units  $(10 \mu\text{g protein})^{-1}$  according to the following formula  $A_{420} \times 1000/\text{min of reaction} \times \mu\text{g of protein} \times 0.1$ . Cell lysates from strain Whetten 1 had no detectable  $\beta$ -gal activity.

## 2.6. Construction of a *fagB(C)* mutant

A Cm resistance cassette from pKRP10 [14] was excised using *Hind*III and cloned into the unique *Cla*I site of pCpO48, which was blunt-ended with T4 DNA polymerase (New England Biolabs) to construct pJGS102. In this construct, the Cm resistance cassette disrupted the *fagB* ORF. pJGS102 was introduced into Whetten 1 by electroporation. The presence of a double reciprocal crossover in a Cm resistant transformant, JGS283, replacing the wild-type *fagB* gene with the insertionally inactivated copy, was confirmed by Southern hybridization.

## 2.7. Virulence studies

Seven weaned, female, Nubian goats, with no history of CL, were inoculated subcutaneously at the lateral aspect of the right distal metatarsus with  $10^7$  CFU of Whetten 1 (three goats) or JGS283 (four goats). After 10 weeks, all goats were killed and specimens collected from the primary lesion and right popliteal lymph node. Samples of the lymph nodes were cultured for bacteria or were fixed in 10% phosphate-buffered formalin, imbedded in paraffin, sectioned and stained with hematoxylin and eosin and examined microscopically for lesions.

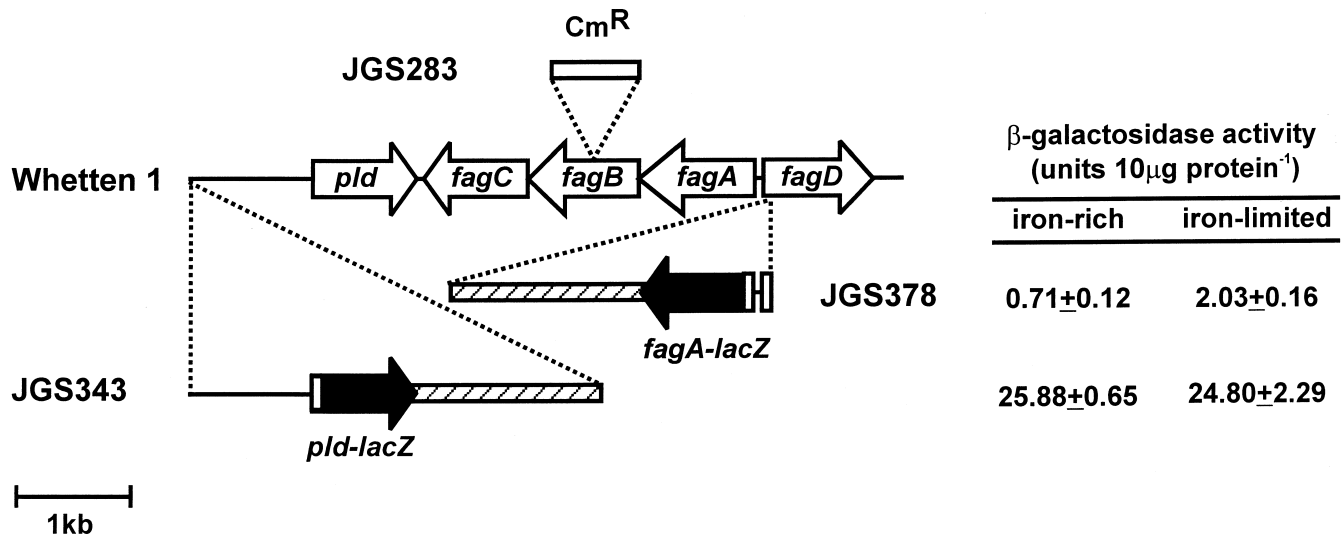


Fig. 1. Organization of the *fag* gene region of *C. pseudotuberculosis*. The *fagA*, *B*, *C*, *D* and *pld* genes are indicated by the arrows. The scale is indicated by the 1-kb bar. The position of the insertion of the Cm resistance cassette in the *fagB(C)* mutant is shown above the map. The *fagA*- and *pld*-*lacZ* fusions are shown below the gene map (only the pJF751 portion of the fusion, indicated by the diagonal hatching, is not shown to scale) and the β-gal activity of each fusion in iron-rich and iron-limited media is shown on the right. β-Gal activities are expressed as the average of three separate experiments ± one standard deviation.

### 3. Results and discussion

#### 3.1. Identification of *C. pseudotuberculosis fag* genes

Analysis of the nucleotide sequence downstream of the *pld* gene indicated the presence of three ORFs transcribed convergently with *pld* (Fig. 1). These ORFs were designated *fag* (Fe acquisition gene) *A*, *B*, and *C*, since they encode proteins which collectively resemble a cytoplasmic membrane transport system for iron-siderophore uptake belonging to the superfamily of ABC transporters. In particular, they show a high degree of similarity to proteins involved in the uptake of the catecholate siderophore, enterochelin (enterobactin) [15,16]. The FagA protein has similarity to the FepD protein of *E. coli* (40.8% identity, 73.5% similarity) and *Yersinia enterocolitica* (39.4% identity, 78.0% similarity), while FagB has similarity to the FepG protein of *E. coli* (44.8% identity, 78.2% similarity) and *Y. enterocolitica* (38.9% identity, 74.9% similarity). The FepD and FepG proteins of the enterochelin uptake

systems are integral membrane proteins which form part of a cytoplasmic membrane permease [15]. Hydropathy profiles of FagA and FagB indicate that these are extremely hydrophobic proteins with nine and seven predicted transmembrane helices, respectively. The third protein, FagC, has similarity to the ATP binding cytoplasmic membrane protein FepC from *Y. enterocolitica* (47.2% identity, 78.1% similarity) and *E. coli* (44.4% identity, 76.0% similarity). Like FepC, FagC has a large ABC transporter domain (InterPro protein [8] database domain: IPR003439) which includes sequences associated with ATP binding. The *fagA*, *B*, and *C* genes are organized head to tail in an operon-like manner. In addition, either the start codon or ribosome binding site of the downstream gene overlaps the stop codon of the preceding gene, suggesting translational coupling of the genes. This organization is very similar to that of the *fepD*, *G*, and *C* genes [15,16]. The putative *fagABC* operon is terminated at a bidirectional terminator located between *pld* and *fagC*.

Table 1

Lesion size, histologic findings and bacterial viable counts of goats challenged with *C. pseudotuberculosis* strains Whetten 1 or JGS283

Strain	Goat	Size of lesion at inoculation site (cm)	Histology <sup>a</sup>	Viable count (CFU g <sup>-1</sup> ) <sup>b</sup>
Whetten 1	29	1.0	++	3.7 × 10 <sup>3</sup>
	40	none	+++	2.3 × 10 <sup>7</sup>
	44	1.0	+++	1.7 × 10 <sup>7</sup>
JGS283	26	none	+	— <sup>c</sup>
	27	1.5	+++	—
	28	1.0	+	—
	32	0.5	+	—

<sup>a</sup>Histologic findings at the popliteal lymph node. +, mild to moderate lymphoid hyperplasia; ++, marked lymphoid hyperplasia; +++, abscessation.

<sup>b</sup>*C. pseudotuberculosis* isolated from the popliteal lymph node.

<sup>c</sup>No bacteria isolated (limits of detection 100 CFU g<sup>-1</sup>).

95-bp upstream of *fagA* and divergently transcribed is a fourth ORF, *fagD*, encoding a protein with similarity to periplasmic iron-siderophore binding proteins (Fig. 1), including the conserved domain for this family of proteins (InterPro database [8] family: IPR002491). In addition, residues 129–144 of FagD had 13 of 16 amino acids in common with the signature sequence of the periplasmic iron complex binding protein superfamily [17]. Unlike the other Fag proteins, which showed similarity to Fep proteins, FagD showed most similarity to the putative iron III citrate binding FecB homologs determined from the genome sequences of *Synechocystis* spp. (34.7% identity, 58.1% similarity) (PIR accession S74441) and *Bacillus subtilis* (34.7% identity, 56.9% similarity) (PIR accession C69805) as well as the iron-regulated SirA lipoprotein from *Staphylococcus aureus* (32.0% identity, 65.6% similarity) [18]. While these proteins tend to be located in the periplasm of Gram-negative bacteria, in Gram-positive bacteria they are usually lipoproteins, associated with the cell wall. Consistent with this location, FagD has a putative signal cleavage and modification site for addition of lipid moieties following translocation across the cytoplasmic membrane. While these results suggest that the *fag* genes encode an iron uptake system, the exact nature of the siderophore involved has not been determined.

The intergenic region between *fagA* and *fagD*, which likely contains the regulatory regions for both the *fagABC* operon and *fagD*, contains the sequence 5'-ttgagtgtgctttcaccacaa-3', which resembles the consensus binding site for the iron response regulator, DtxR [19], with 10 of 19 consensus residues.

### 3.2. Induction of the *fag* genes by limited iron

To test the ability of the putative *fagABC* operon to be induced by limited iron, a *lacZ* fusion to the beginning of *fagA* was introduced into the Whetten 1 chromosome to create JGS378, as described in Section 2.5. As a positive control for the  $\beta$ -gal assays, a *pld-lacZ* fusion was also introduced into Whetten 1, to create JGS343. All strains were created by single crossover events, such that the promoter region was duplicated and no genes were disrupted. JGS343 and JGS378 were grown in both iron-rich and iron-limited media and assayed for  $\beta$ -gal activity (Fig. 1). JGS343 showed high levels of  $\beta$ -gal activity in either media, confirming that *pld* expression is not regulated by iron. In contrast, JGS378 expressed very low levels of  $\beta$ -gal activity in iron-rich media and these levels were increased almost three-fold in iron-limited media. These results suggest that the *fag* operon was not well expressed in vitro, but did appear to be induced by limited iron.

### 3.3. Utilization of iron sources by a *C. pseudotuberculosis fagB(C)* mutant

To access whether the *fag* genes were required for the

utilization of iron sources by *C. pseudotuberculosis*, a Cm resistance cassette was used to disrupt the *fagB* ORF and the mutated allele introduced onto the chromosome of Whetten 1, to create JGS283. This mutation disrupted expression of *fagB*, and likely the downstream gene, *fagC*. Since little is known about the use of iron sources by *C. pseudotuberculosis*, Whetten 1 and JGS283 were examined for their ability to acquire iron from a variety of sources. Both Whetten 1 and JGS283 were able to use FeCl<sub>3</sub>, FeSO<sub>4</sub>, hemin, hemoglobin and transferrin for growth when plated on BHI agar supplemented with 50  $\mu$ M EDDHA, indicating that there was no defect in the utilization of any of these compounds by JGS283. Given the redundancy of iron utilization systems in some bacteria, it is possible that the effect of the Fag uptake system is masked by another uptake system with similarity specificity. It is also possible that the *fag* genes are responsible for the uptake of a siderophore which is not produced by *C. pseudotuberculosis*, but which *C. pseudotuberculosis* can scavenge in vivo, from other bacteria. This latter scenario is observed with organisms such as *Campylobacter* species [20] and may be supported by the lack of siderophore biosynthetic genes clustered with the *fag* genes.

### 3.4. A *fagB(C)* mutant is reduced for virulence in goats

To test whether the *fag* genes were required for the virulence of *C. pseudotuberculosis*, Whetten 1 and its isogenic *fagB(C)* mutant were tested for virulence in a goat model of CL (Table 1). Bacteria were isolated in high numbers from the popliteal lymph node of each of the three goats inoculated with Whetten 1, with abscessation observed in most cases. In contrast, only one of four goats inoculated with JGS283 showed any abscessation, and no bacteria were recovered from the popliteal lymph nodes of these animals. Interestingly, little difference was seen between the two strains at the injection site where abscesses can develop from deposited bacteria. These data suggest a decreased ability of the *fagB(C)* mutant to survive once in the body and is consistent with a defect in iron scavenging ability, since the body represents a low iron environment. These results also suggest that, despite the low level of expression of the *fag* genes observed in vitro, *fag* genes are expressed in vivo to a level required for full virulence. It is possible that the *fag* genes require another factor found in vivo, in concert with iron limitation, to achieve full induction.

In conclusion, we have identified a putative *C. pseudotuberculosis* iron uptake gene cluster consisting of *fagABC* and *fagD*. Although these genes are not well expressed in vitro, their expression appears to be regulated by iron. While no defect in iron utilization in vitro could be identified in a *fagB(C)* mutant, expression of these genes in vivo appears to contribute to the virulence of *C. pseudotuberculosis* in a goat model of CL.

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