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Contribution of the FeoB transporter to *Streptococcus suis* virulence

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Summary. The contribution of iron transporter systems encoded by *feo* genes to the pathogenic traits of streptococci is largely unknown, despite the fact that those systems are required for the full virulence of several gram-negative bacterial species. In this work, we show that the swine pathogen and zoonotic agent *Streptococcus suis* has a *feoAB* operon similar to that encoding an iron transporter system in *Escherichia coli*. Electrophoretic mobility assays and transcriptional analyses confirmed that the expression of *S. suis feo* genes is under the negative control of the ferric uptake regulator (Fur) protein. In vivo trials in mice using a *feoB* defective mutant strain were carried out to investigate the contribution of this gene to the virulence of *S. suis*. The results showed that the median lethal dose (LD_{50}) of the mutant was approximately 10-fold higher than that of the wild-type parent strain. These data suggest that the Feo metal transporter plays a significant role in streptococcal infectious disease. This is in contrast to previous results reported for this same gene in other gram-positive bacterial species. [**Int Microbiol** 2009; 12(2):137-141]

Keywords: *Streptococcus suis* · operon *feo* · iron uptake · bacterial virulence · streptococcal diseases

Introduction

Streptococcus suis is a major pathogen of swine that is responsible for severe economic losses in the pork industry [13]. In addition, this bacterium is an emerging zoonotic agent, considered to be the primary cause of adult meningitis in Vietnam and the second highest cause of this disease in Thailand [17,26]. Moreover, in a recent outbreak in China, more than 200 human cases of *S. suis* infection, resulting in 39 deaths, were reported [12]. In both pigs and humans, the main clinical manifestations of *S. suis* infection are septicemia and meningitis [12].

Iron is an essential cation for living organisms, although its availability in the body fluids of host organisms is extremely low [1]. To cope with this scarcity, most bacterial pathogens have evolved many different iron uptake systems [30]. In *Escherichia coli*, for example, the Fe^{2+} transport system is encoded by *feo* genes, which act in concert to maintain cellular iron supplies under anoxic conditions [15,25].

In *Escherichia coli*, the *feoABC* operon encodes three predicted proteins: FeoA, a small 75-residue hydrophilic protein of unknown function; FeoB, a large 773-residue protein with an integral membrane domain likely to act as the ferrous permease; and FeoC, a small 78-residue hydrophilic protein that has been suggested to function as a transcriptional repressor, so far only associated with gammaproteobacterial *feo* systems [7,20]. FeoB homologs are found in both gram-negative and gram-positive bacteria [20]. Interestingly, it has been shown that these homologs contribute to the virulence of gram-negative organisms, such as *E. coli* [25], *Helicobacter pylori* [29], *Campylobacter jejuni* [20], *Porphyromonas gingivalis* [8], and *Legionella pneumophila* [22]. However, FeoB involvement in the virulence of gram-positive microorganisms has been analyzed only in *Listeria monocytogenes*.

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In contrast to previous reports in gram-negative bacteria, the virulence of *L. monocytogenes feoB* mutants in mice is not affected [14].

To our knowledge, the contribution of *feo* genes to the virulence of members of the *Streptococcus* genus has never been reported. In this work, *in silico* analysis was used to identify a *feoAB* operon in *S. suis*. We show that this operon is under the control of the ferric uptake regulator Fur, and that the product of the *feoB* gene is a significant virulence factor for *S. suis*.

Materials and methods

Bacterial strains, plasmids, and molecular techniques. The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth or agar [19]. When necessary, ampicillin (50 μg/ml), chloramphenicol (34 μg/ml), and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Roche) were added to the growth media. *Streptococcus suis* was grown in Todd-Hewitt medium (TH; Difco). Growth under metal-depleted conditions was achieved by the addition of the metal chelator 2,2´-dipyridyl (DPD; Sigma), at a final concentration of 100 μM, to TH broth 30 min before the inoculation of *S. suis* cells. When required, spectinomycin (100 μg/ml) and chloramphenicol (5 μg/ml) were added to TH medium. DNA extraction, plasmid mini-preparations, cloning, and transformation were carried out using standard procedures [24].

Construction of the *Streptococcus suis feoB* mutant. *Streptococcus suis* UA5003 mutant (*feoB*::*spc*) was obtained by insertional mutagenesis using allelic replacement. Briefly, the *feoB* gene of *S. suis* strain P 1/7 was PCR-amplified using Pwo DNA polymerase (Roche) and primers 1638upSma and 1638dwSma (Table 2), which introduce *Sma* I restriction sites at both ends. The amplicon was cloned into the pGEM-T vector (Promega). Afterwards, the entire construction was amplified by overlap extension PCR with primers 1638intUP and 1638intDW (Table 2), which anneal to an internal region of the *feoB* gene. A spectinomycin (*spc*) cassette released from pR412 [18] was treated to generate blunt ends and then ligated into the purified PCR product using T4 DNA ligase (Roche). After the resulting *feoB*::*spc* fragment had been digested with *Sma* I, the inactivated gene was cloned into the thermosensitive suicide vector pSET5s [28], giving rise to plasmid pSET5s*feoB*::*spc*. This vector was propagated in *E. coli* DH5α cells (Clontech) according to the manufacturer's instructions, purified, and introduced into *S. suis* strain P1/7 by electroporation [27]. Allelic replacement procedures were carried out as described previously [3]. Gene replacement in candidate clones was confirmed by PCR and by sequencing of the region in the resulting mutant using primers SecAup2 and SecAup3 (Macrogen Sequencing Service) (Table 2).

Virulence assays. All animal experiments were approved by the UAB Animal Ethics Committee. Female BALB/cAnNHsd mice (8-week old) obtained from Harlan Iberica (Barcelona, Spain) were used for virulence assays [2]. Four groups of three mice were used for each of the wild-type (WT) P1/7 or mutant UA5003 (*feoB*::*spc*) strains. Animals were injected intraperitoneally as described [11] with 0.1 ml of serial 10-fold dilutions of bacteria in suspensions of TH broth, supplemented with 10% inactivated bovine serum (Invitrogen). The concentrations of the original bacterial suspensions were determined by the plate count method. Animals were followed for 3 weeks post-inoculation. Mortality was recorded, and the lethal dose 50 (LD_{50}) was calculated as previously described [4].

RNA techniques. RNA was isolated as previously described [10], with slight modifications. Ten ml of a *S. suis* culture in the mid-exponential growth phase (OD_{600} = 0.6) were collected by centrifugation at 8000 ×*g* for 10 min. Cells were then resuspended by vigorous shaking at 37ºC for 10 min in 300 μl of Tris-EDTA pre-lysis buffer containing 10 mg of lysozyme per ml. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Contaminating DNA was removed from the RNA during purification by treatment with RNase-free DNase (Qiagen) followed by digestion with DNase Turbo (Ambion). The concentration and integrity of RNA were determined by measuring the absorbance at 260 nm (A_{260}) and by 1% agarose gel electrophoresis, respectively. In all cases, the absence of DNA in RNA samples was confirmed by PCR without the addition of reverse transcriptase. Reverse transcription-PCR (RT-PCR)

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant features	Source or reference
Strains		
Streptococcus suis		
P1/7	Virulent serotype 2 strain	
UA5001	Derived from P1/7. Δ*fur*	[3]
UA5003	Derived from P1/7.*feoB*::*spc*. Spc^r	This work
Escherichia coli		
BL21-CodonPlus(DE3)-RIL	B F^- *ompT hsdS* (r_B m_B^-) dcm^+ Tc^r *gall* (DE3) *endA* The [*argU ileY leuW* Cam^r]	Stratagene
DH5α	*supE4* Δ*lacU169* (Φ80 *lacZ*ΔM15) *hsdR recA1 endA1 gyrA96 thi-1 relA1*	Clontech
Plasmids		
pGEM-T	PCR cloning vector. Ap^r	Promega
pET15b	His_6 tag expression vector. Ap^r	Novagen
pR412	pEMcat derivative. Ap^r, Spc^r	[18]
pSET5s	Temperature-sensitive suicide vector for *S. suis* mutagenesis. Cat^r	[28]
pSET5s*feoB*::*spc*	pSET5s vector carrying the construction for *feoB* mutagenesis	This work

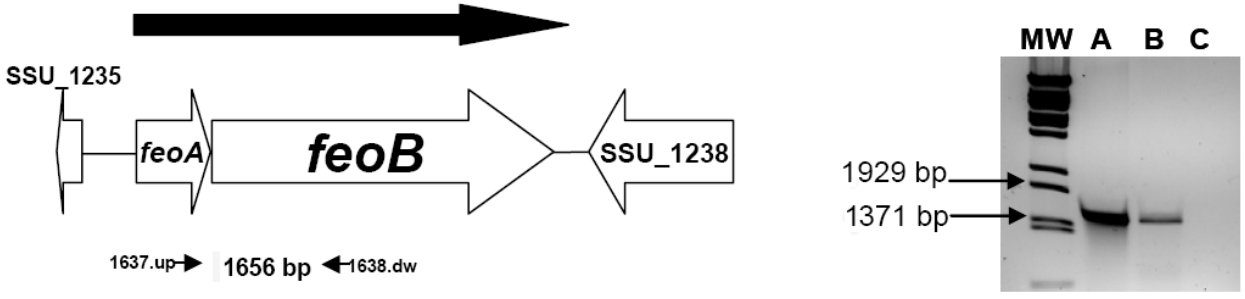
Table 2. Oligonucleotide primers used in this work

Oligonucleotide	Sequence (5′–3′)	Application
1638intUP	CTAGATTTGCCTGAATAAGG	Primer used to construct the pSET5*sfeoB*::*spc* plasmid
1638intDW	CTTGATGGCATTATTGCAGG	Primer used to construct the pSET5*sfeoB*::*spc* plasmid
1638upSma	CCCGGGATCTGCAAAACCTTCAAGTTGG	Primer used to construct the pSET5*sfeoB*::*spc* plasmid
1638dwSma	CCCGGGATTCGGTAAATGCTAGTCTTG C	Primer used to construct the pSET5*sfeoB*::*spc* plasmid
SecAup2	TTCCAACGCCAAACTGG	Primer used to confirm *feoB* mutant
SecAup3	ACCGACTGAGCTAAACCAG	Primer used to confirm *feoB* mutant
M13F	GTTTTCCCAGTCACGAC	Forward primer to amplify from pGEM-T vector. DIG-labelled at its 5′ end to obtain EMSA probes
M13R	CAGGAAACAGCTATGAC	Reverse primer to amplify from pGEM-T vector. DIG-labelled at its 5′end to obtain EMSA probes
T7 Promoter	TAATACGACTCACTATAGGG	Forward primer for sequencing pET15b vector
T7 Terminator	GCTAGTTATTGCTCAGCGG	Reverse primer for sequencing pET15b vector
1637.up	ACACTTAGCGCACTTGGGTTTA	Upper primer for real-time quantitative RT-PCR assay to determine *feoA* gene expression and the upper primer used in RT-PCR assay
1637.dw	GCCTAGGGGAGCGACTTTTT	Lower primer for real-time quantitative RT-PCR assay to determine *feoA* gene expression
1638.up	CTGGCTGGCTACAATCTCTGGT	Upper primer for real-time quantitative RT-PCR assay to determine *feoB* gene expression
1638.dw	TTTTCGGTCCTGTTCATTTTCA	Lower primer for real-time quantitative RT-PCR assay to determine *feoB* gene expression and the lower primer used in RT-PCR assay
cys.up	GGTAACACTGGTATCGGTCTTG	Forward primer for real-time quantitative RT-PCR assay of the cysteine synthase gene
cys.dw	CCTGTCCTGTTGTATCTTCGTG	Reverse primer for real-time quantitative RT-PCR assay of the cysteine synthase gene
1637.pr	TCCTCTCTAGGTGTTAGC	Forward primer to obtain EMSA probes of the feoAB promoter region
1637.50	TATCATCTTGCGTTAGGC	Reverse primer to obtain EMSA probes of the *feoAB* promoter region
fur.up.NdeI	ACTGACATATGGAACTCCATTCTCACTT CAATGC	Upper primer for cloning the *S. suis fur* gene into the pET15b expression vector
fur.rv.BamHI	AGGGATCCATTCGTCTTTTAGCCCTGAC	Lower primer for cloning the *S. suis fur* gene into the pET15b expression vector

assays were done using a Titan One Tube RT-PCR system (Roche) and following the manufacturer's instructions. Real-time quantitative RT-PCR (rRT-PCR) analysis of total RNA was performed for all genes, as reported previously [6], using specific internal oligonucleotides for each gene (Table 2). The cysteine synthase gene was used as a standard, since its expression was not affected under the evaluated conditions. The total RNA concentrations of the WT and *fur* mutant strains were adjusted to the same value, and the genes to be tested, as well as the standard, were assayed simultaneously using a set of standard samples in each case.

Protein overexpression. The *S. suis* Fur protein was overexpressed as follows. The DNA fragment containing the *fur* gene was amplified from purified chromosomal DNA of *S. suis* P1/7 strain by PCR, using the primers fur.up.NdeI and fur.rv.BamHI (Table 2). The purified PCR product was then enzymatically digested with the corresponding restriction enzymes, cloned into the pET15b expression vector, and transformed into *E. coli* DH5α cells. Recombinant plasmids were predicted to express an N-terminal His6-tagged fusion protein, and correct in-frame fusions of the protein into pET15b were confirmed by sequencing of plasmid DNA with T7 promoter and T7 terminator primers (Macrogen Sequencing Service) (Table 2). Recombinant plasmids were then used to transform the BL21-CodonPlus (DE3)-RIL strain. An overnight culture of the resulting transformants was diluted (1/20) in 10 ml of LB medium and incubated at 37°C until OD_{600} 0.6 was reached. Expression of the fusion protein was induced at this time by the addition of IPTG to a final concentration of 1 mM. After incubation for an additional 3 h at 37°C, the cells were collected by centrifugation at 8000 ×*g* for 10 min and resuspended in 20 mM Tris-HCl (pH 8), 50 mM KCl, 5% (vol/vol) glycerol, and a Complete Mini protease inhibitor cocktail (Roche). The cell suspension was then lysed by sonication on ice for 5 min at 50 W using a Braun LabsonicU (Braun Biotech). Unbroken cells and debris were removed by centrifugation at 7000 ×*g* for 10 min. Crude extracts were visualized by SDS-PAGE [16] and stored at 4ºC for electrophoretic mobility shift assays .

Electrophoretic mobility shift assays (EMSA). EMSA were done as previously described [5] with slight modifications. The *feo* promoter was PCR-amplified from P1/7 *S. suis* genomic DNA using oligonucleotides 1637.pr and 1637.50 (Table 2). The obtained DNA fragment (396 bp), extending from position +67 to –329 (with respect to its predicted transla-



ORF annotation	Length product (aa)	Putative function
SSU_1235	42	Hypothetical protein
SSU_1236	156	Fe^{2+} transporter component (FeoA)
SSU_1237	714	Fe^{2+} transporter component (FeoB)
SSU_1238	312	ATPase involved in cell division

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Fig. 1. Genetic organization of *Streptococcus suis* P1/7 strain chromosomal region containing the *feoAB* genes, based on [http://www.sanger.ac.uk/Projects /S_suis/]. The large black arrow indicates the putative transcriptional unit that was tested. The primer sets used for the RT-PCR assays are indicated by small arrows. RT-PCR was carried out in the presence of total RNA (lane A), DNA (lane B), or in the absence of both RNA and DNA (lane C). *Bst*EII-digested-λ DNA was used as a molecular size marker (MW). The lengths of the gene products in number of amino acids and the putative functions of the genes are also indicated.

tional starting point), was cloned in *E. coli* DH5α cells through the pGEM-T vector. The presence of the desired promoter was confirmed by sequencing the plasmid DNA using the primers M13F and M13R (Table 2). DNA probes were prepared by PCR amplification, with one of the oligonucleotides labeled with digoxigenin (DIG) at its 5´ end. DNA-protein reaction mixtures (20 µl) containing 25 ng of a DIG-DNA-labeled probe and different concentrations of either crude extracts overexpressing or not the *S. suis* Fur protein were incubated in EMSA buffer [20 mM Tris-HCl (pH 8), 50 mM KCl, 5% (vol/vol) glycerol, 1 µg bulk carrier sperm salmon DNA, 0.5 mM 1,4-dithiothreitol, and 0.1 mg bovine serum albumin per ml] for 10 min at room temperature. DNA-protein complexes were visualized by separation on a 5% nondenaturing polyacrylamide gel (40 mM Tris-acetate) at 150 V for 1.5 h, and then transferred to a Biodine B nylon membrane (Pall Gelman Laboratory). DIG-DNA-labeled-protein complexes were detected according to the manufacturer's protocol (Roche).

Results and Discussion

The upstream region of the *E. coli feo* operon contains a binding site for the Fur protein. Together with iron (Fe^{2+}), Fur acts as a corepressor [15]. In a previous work, the *E. coli* (*fur*) homologue was identified in the *S. suis* P1/7 strain through bioinformatics analysis [3]. In this study, further *in silico* searches in the database containing the complete annotation of the *S. suis* P1/7 strain genome [http://www.sanger.ac.uk /Projects/S_suis/] were carried out using the TBLASTX program and the *E. coli feoA* gene sequence as probe.

An open reading frame (ORF) corresponding to a protein of 156 amino acid residues and homologous to the *E. coli feoA* gene was found to be encoded by the *S. suis* P1/7 strain SSU_1236 gene. The calculated molecular mass of the predicted translated *S. suis* FeoA protein was 17,433 Da. Immediately downstream of the SSU_1236 gene, another ORF (encoded by gene SSU_1237), homologous to the *E. coli feoB* gene, was identified (Fig. 1). The predicted translated FeoB protein (714 amino acid residues) had a calculated molecular mass of 79,083 Da. In contrast to the organization of the gene in *E. coli*, no *feoC* homologues were found in the *S. suis* genome. The identity between the *S. suis* FeoA and FeoB proteins and the protein products encoded by the *E. coli feoA* and *feoB* genes was 37% and 35%, respectively. RT-PCR analysis of the transcriptional pattern of the *S. suis feoA*B genes showed that they belong to the same transcriptional unit (Fig. 1). A similar transcriptional organization of the *feo* genes is found in *L. monocytogenes* [14,15].

In silico analysis of the upstream regions of *feo* operons from multiple proteobacterial genomes have revealed a conserved palindromic motif corresponding to a Fur binding site [9,23]. However, no apparent conserved motifs were found in the promoter region of the *feoAB S. suis* operon. Since in *E. coli* the *feoABC* operon is under the control of Fur [15], we overexpressed the *S. suis* Fur protein (Fig. 2A) and then used

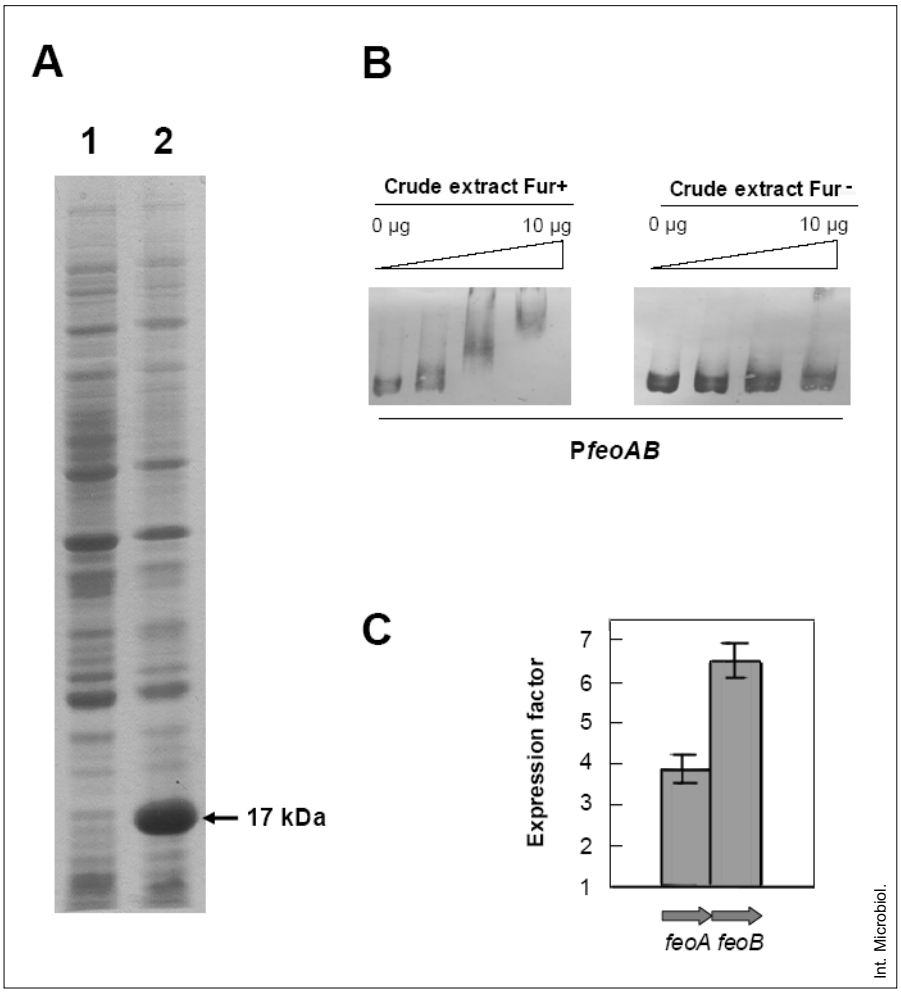

Fig. 2. (**A**) SDS-PAGE profile of a crude extract from *Escherichia coli* BL21-CodonPlus(DE3)-RIL cells containing the pET15b vector without (lane 1) or with (lane 2) overexpression of the *Streptococcus suis* Fur 17-kDa protein. (**B**) Electrophoretic mobility of DNA fragments containing the *S. suis feoAB* promoter. The analyses were carried out in the presence of increasing amounts (0–10 µg) of crude extract from *E. coli* BL21-CodonPlus(DE3)-RIL cells containing (left) or not (right) the pET15b vector overexpressing the *S. suis* Fur protein. All EMSA were repeated a minimum of three times to ensure the reproducibility of the results. (**C**) Up-regulation of *feoA* and *feoB* genes in the *S. suis fur* mutant (UA5001) as measured by real-time quantitative RT-PCR. The expression factor is the ratio of the mRNA concentration of each gene from UA5001 strain with respect to the WT parent strain P1/7 (defined as 1). The relative mRNA concentration of each gene was determined by using a standard curve generated by amplifying an internal fragment of the *S. suis* P1/7 ORF encoding the cysteine synthase enzyme. The mean value and the standard deviation from two independent experiments (each in duplicate) are also indicated.

EMSA studies to test its ability to bind the promoter region of the *feoAB* transcriptional unit. Interestingly, the EMSA results showed that *S. suis* Fur binds to the *feoAB* promoter (Fig. 2B). Binding was specific since the presence of unrelated DNA as a competitor did not abolish the Fur-mediated retarding band (Fig. 2B). In agreement with these data, derepression of *feoA* and *feoB* gene expression was observed in the *S. suis fur* mutant UA5001 [3] through real-time quantitative RT-PCR assays (Fig. 2C).

In order to study the contribution of the *feoAB* operon to *S. suis* virulence, the *S. suis feoB* gene was inactivated in the P1/7 virulent strain, giving rise to mutant strain UA5003 *feoB*::*spc* (Table 1). The virulence of this strain was subsequently evaluated in a murine model of infection, comparing the LD_{50} of the *feoB::spc* mutant to that of the WT parent strain P1/7. Four groups of three female 8-week-old BALB/cAnNHsd mice were injected intraperitoneally with 0.1 ml of serial 10-fold dilutions of bacteria prepared from each bacterial strain. This assay showed that the virulence of the *feoB* mutant ($LD_{50} = 3.9 \times 10^7$ CFU/animal) was significantly attenuated compared with that of the WT strain ($LD_{50} = 4.4 \times 10^6$ CFU/animal). Note that the in vitro growth rates of the *S. suis feoB* mutant and the WT strains did not differ under the metal-repleted conditions present in the TH medium (Fig. 3). However, in the presence of the chelator DPD, the growth rate of the *feoB* mutant was lower than that of the wild-type P1/7 strain. This behavior can be attributed to a decreased capacity of the FeoB-deficient mutant to grow under metal-depleted conditions.

Streptococcus suis has been shown to not require iron for growth in vitro [21]. In addition, the degree of identity between FeoB from *S. suis* and the *E. coli* Feo transporter is low, and some FeoB homologues transport manganese rather than iron [8]. However, the impaired virulence of the *S. suis feoB* mutant, together with the fact that the genes encoding the Feo transporter are Fur regulated, indicate that, under the low concentrations of these elements existing inside host cells and in mammalian body fluids, the *feoB*-encoded protein of *S. suis* might act as an important transporter for metal uptake in vivo. Furthermore, it has been reported that the

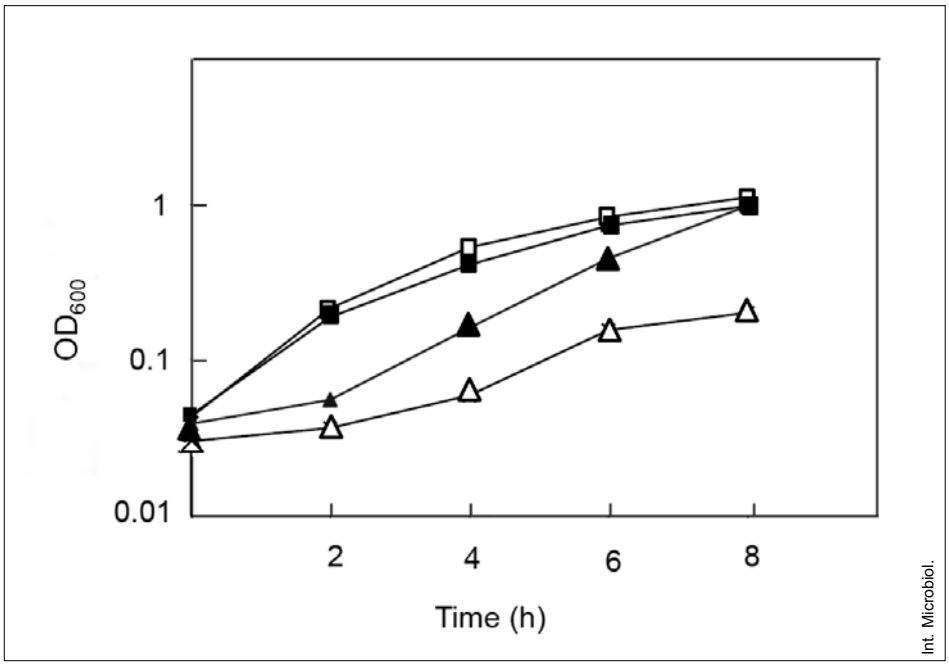

Fig. 3. Growth of wild-type P1/7 (open squares) and UA5003 *feoB* mutant (closed squares) of *Streptococcus suis* strains in TH medium; and the same strains, P1/7 (closed triangles) and UA5003 (open triangles), in TH medium in the presence of the metal chelator DPD (100 μM). The means from three independent experiments (for each growth curve) are represented. For simplification, error bars have been omitted; the standard deviation was <10 % in all cases.

FeoB transporter is required for the intracellular growth of *Legionella pneumophila* [22] and for the virulence of several gram-negative bacteria [8,20,22,25,29].

To summarize, the data reported in this work demonstrate that full virulence of *S. suis* requires the FeoB transporter. To our knowledge, this study is the first to describe the contribution of the *feoB* gene to the virulence of gram-positive bacterial pathogens.

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