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Brucella abortus efp gene is required for an efficient internalization in HeLa cells

Florencia Iannino, Juan E. Ugalde, Nora Iñón de Iannino*

Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH), Consejo de Investigaciones Científicas y Técnicas, Universidad Nacional de San Martín (CONICET-UNSAM), Av. Gral. Paz 5445, Buenos Aires, Argentina

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ABSTRACT

Numerous chromosomal virulence genes (*chv*) have been shown to play an important role in the ability of *Agrobacterium tumefaciens* to transform plants. The *A. tumefaciens chvH* gene encodes a protein similar in sequence to the *Escherichia coli* elongation factor P (EF-P). In *A. tumefaciens* this factor is required for tumor formation and for full expression of the *vir* genes, exerting its activity at a post-transcriptional level. Cross-complementation assays suggest that the *chvH* gene and the *efp* gene of *E. coli* are functionally homologous. We have cloned and characterized the *efp* homolog gene in *Brucella abortus* which has 45% identity to *A. tumefaciens chvH* and 35% identity to *E. coli efp*. The gene complemented detergent sensitivity and virulence in the *chvH A. tumefaciens* mutant, suggesting that both genes are functionally homologous; the growth rate in complex medium also increased to wild type levels. An *efp* mutant in *B. abortus* 2308 grew slower in complex media and showed more sensitivity to detergents. Infection assays in J774 macrophage like cells revealed no significant differences between the wild type and the *efp* mutant strains. The recovery of this mutant from spleens of inoculated mice was equivalent compared to that of the parental strain suggesting that *B. abortus efp* is not required for virulence in an animal model. However the *efp* mutant revealed significant differences at 1 h–4 h post-infection in HeLa infection assays compared to the wild type strain, indicating that cellular internalization was affected in non-professional phagocytes. Double immunofluorescence assays for detecting extracellular and intracellular bacteria, demonstrated that the mutant attaches to HeLa cells as the wild type but is deficient in the internalization process, thus indicating that *efp* is involved in the penetration of *Brucella* in non-professional phagocytes.

1. Introduction

Brucella abortus is an intracellular pathogen that causes brucellosis, a world-wide zoonosis [1] that affects bovines and has the capacity to infect humans. While in bovines the main manifestation is abortion, in humans is a serious disease characterized by different manifestations like undulant fever, endocarditis, osteoarticular complications, and several neurological disorders.

Brucellosis in humans is primarily a disease of the reticuloendothelial system since the pathogen has the capacity to multiply inside phagocytic cells such as macrophages and non-professional phagocytic cells [2,3]. The bacteria infect the host through the mucosal barriers and, thus, the interaction with host epithelial cells at these locations represent an important point of initial contact between the pathogen and the host.

The analyses of the genome of *B. abortus*, *Brucella melitensis* and *Brucella suis* revealed that pathogenic islands, the complete set of genes required for types I, II and III secretion systems, and the classical virulence factors are absent in *Brucella* spp. [4].

Brucella spp., as *Agrobacterium* spp. and *Rhizobium* spp., belong to the alpha-2 subgroup of *Proteobacteria* according to 16S rRNA sequence [5]. Most genus of this group are characterized by their ability to interact with eukaryotic cells as endosymbionts or pathogens [6] and a number of similar chromosomal loci are known to be involved in the success of these interactions. One of such conserved systems is the two-component regulatory system BvrR–BvrS involved in virulence, cell invasion and intracellular replication in *B. abortus* [7]. This system is highly similar to the *Sinorhizobium meliloti* ChvI–ExoS and *Agrobacterium tumefaciens* ChvI–ChvG, both shown to be critical for endosymbiosis and pathogenicity in plants, respectively [8,9]. Another highly conserved virulence factor is the type IV secretion system of *Brucella*, essential for the intracellular trafficking of the bacteria in host cells [10,11] which is highly similar to the *virB* operon of *A. tumefaciens* that mediates the transfer of the T-DNA into the

* Corresponding author. Instituto de Investigaciones Biotecnológicas, Universidad Nacional de Gral. San Martín (IIB-UNSAM), Av. Gral. Paz 5445, INTI, Ed. 24, CP1650, San Martín, Buenos Aires, Argentina. Tel.: +54 11 4580 7255; fax: +54 11 4752 9639.
E-mail address: norai@iib.unsam.edu.ar (N. Iñón de Iannino).

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plant cell and drives transformation. The *A. tumefaciens chvA*/*chvB* genes (coding for the cyclic β-1,2-glucan synthase and the ABC transporter of glucans to the periplasm respectively) and their equivalent *ndvA*/*ndvB* genes of *S. meliloti* and *cgt*/*cgs* of *B. abortus* [12-15] are other examples of conserved functions between bacteria with different pathogenic or symbiotic lifestyles. While mutants in the *chvB*/*chvA* genes render *Agrobacterium* avirulent [16,17], mutants in the *S. meliloti ndvB* or *B. abortus cgs* genes form ineffective pseudonodules in alfalfa or display reduced virulence in mice, respectively [18,19].

Mutational studies identified a number of chromosomal virulence genes (*chv*) that play important roles in the virulence of *A. tumefaciens*; however, the role of these genes in the bacteria–host interaction is difficult to assess because all these mutants show a pleiotropic phenotype and have several altered cell surface properties, like increased sensitivity to certain antibiotics and detergents [20]. The *A. tumefaciens chvH* locus, shown to be required for tumor formation [8], encodes a protein with a 57% similarity to the elongation factor P of *Escherichia coli*, a protein thought to be involved in peptide bond synthesis [21,22]. Protein translation is dependent on a complete set of translation factors and the elongation factor P (EF-P) is one of these factors. It is strictly conserved in bacteria and has been shown to have a role in translation initiation by promoting the formation of the first peptide bond [23]. Under certain conditions, the *efp* gene of *E. coli* is essential for cell viability [24]. Homologs of EF-P are present in all kingdoms of life (called eIF5A in eukaryotes) and complementation assays suggest that the *A. tumefaciens chvH* gene is functionally homologous to the *E. coli efp* gene. The wild type *chvH* locus is essential for full expression of *vir* genes encoded by the Ti plasmid, regulation that is exerted at a post-transcriptional level [25].

In this work we describe the cloning and the mutagenesis of *B. abortus efp* gene highly similar to *A. tumefaciens chvH* and *E. coli efp* genes. The characterization of the mutant in the intracellular multiplication and in the mouse models is reported.

2. Materials and methods

2.1. Bacterial strains, plasmids and media growth conditions

All live *Brucella* strains have been manipulated in a BSL-3 facility at UNSAM University. The bacterial strains and plasmids used in this study are listed in Table 1. *A. tumefaciens* was grown at 28 °C in tryptone yeast medium (TY) [26]. *Brucella* strains were grown in brucella broth (BB, Difco Laboratories, Detroit, Mich.) on rotary shaker (250 rpm) at 37 °C. *E. coli* strains were grown at 37 °C in Luria Bertani broth (LB) [27]. When necessary, the following antibiotics were added to the indicated final concentrations: for *A. tumefaciens*, kanamycin (100 μg/ml) and ampicillin (50–100 μg/ml); and for *Brucella*, nalidixic acid (5 μg/ml), kanamycin (50 μg/ml) and ampicillin (50 μg/ml). *A. tumefaciens* A6880 and plasmid pWT136 were provided by Dr. E. Nester (Dept. Microbiology, Washington University). The construction of new strains and plasmids is described below in the text.

2.2. Cloning, nucleotide sequence and mutagenesis of efp gene

A PCR product of 1.3-kb containing the *efp* gene (BAB1_1722) was amplified from *B. abortus* 2308 chromosomic DNA using primers 5′ CGACGACCAGATAAAGCA 3′ and 5′ CGATGCTGTCCGTCCTTG 3′. The fragment obtained was ligated to pGem-T-Easy (Promega corp.) to generate pGemT-*efp*. This plasmid was sequenced with T7 or SP6 primers using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and was analyzed in an ABI Prism 377 sequencer (Applied Biosystems). The sequences were compared against the National Center for Biotechnology Information non redundant protein database by using the BLASTx or BLASTp programs on the BLAST network service.

A 0.5-kb *Nar*I fragment was deleted within the *efp* coding region from the pGemT-*efp*. The resulting plasmid was treated with T4 DNA polymerase (NEB, Inc.) and then ligated to a 1.2-kb. *Hinc*II fragment containing a kanamycin resistant cassette to generate the plasmid pGemT-*efp*::Km. This plasmid was electroporated into *B. abortus* 2308 and double crossover events (Km^r Amp^s) were selected using kanamycin resistance. Colony PCR analysis showed that the wild type *efp* gene had been replaced by the disrupted one. The strain was designated as *B. abortus efp*.

2.3. Complementation of B. abortus efp mutant

The *B. abortus efp* strain was complemented using biparental mating with S17.1(λpir) strain containing the plasmid pBBR1MCS-4

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Phenotype or genotype[a]	Reference or resource
Strain		
A. tumefaciens		
A348	A136(pTiA6), wild type	Laboratory stock
A6880	A6007 *chvH*::T*nphoA*	Provided by Dr. Nester
A6880(pBBR4*efp*)	A6007 *chvH* mutant harboring plasmid pBBR4*efp*	This study
A6880(pBBR4*chvH*)	A6007 *chvH* mutant harboring plasmid pBBR4*chvH*	This study
E. coli		
XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*) *173 endA1 supE44 thi-1 recA1* gyrA96 relA1 lac *[F′* proAB $lacI^qZ\Delta M15$ *Tn*10, Tet^r	Stratagene
S17.1(λpir)	λ lysogenic S17-1 derivative producing π protein for replication of plasmids carrying oriR6 K, Nal^s	[36]
HB101(pRK2013)	Triparental conjugation helper strain; Km^r	[29]
B. abortus		
2308	Virulent, field isolated, wild type, Nal^r	Laboratory stock
efp	2308 *efp* mutant, *efp*:: Km^r	This study
efp(pBBR4*efp*)	2308 *efp* mutant harboring plasmid pBBR4*efp*, Km^r Amp^r	This study
efp(pBBR4*chvH*)	2308 *efp* mutant harboring plasmid pBBR4*chvH*, Km^r Amp^r	This study
Plasmid		
pWT136	2-kb *Eco*RI fragment containing the *A. tumefaciens chvH* gene cloned into pBluescript II KS +	Provided by Dr. Nester
pGemT-easy	Cloning vector for PCR products, Amp^r	Promega
pGemT-*efp*	1.3-kb PCR product containing the *B. abortus* 2308 *efp* gene cloned into pGemT-easy	This study
PUC4 K-Km	Source of kanamycin resistance cassette	Laboratory stock
pGemT-*efp*::Km	pGemT-*efp* with a 0.5-kb *Nar*I deletion and containing a kanamycin resistance cassette	This study
pBBR1MCS-4	Broad-host-range cloning vector, Amp^r	[28]
pBBR4*efp*	1.3-kb *Eco*RI fragment containing the *B. abortus* 2308 *efp* gene cloned into pBBR1MCS-4	This study
pBBR4*chvH*	2-kb *Eco*RI fragment containing the *A. tumefaciens chvH* gene cloned into pBBR1MCS-4	This study

[a] Abbreviations: Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance; Sm^r, streptomycin resistance; Tet^r, tetracycline resistance.