

# IDENTIFICATION OF TWO SUBSTRATES OF FTS\_1067 PROTEIN – AN ESSENTIAL VIRULENCE FACTOR OF *FRANCISELLA TULARENSIS*

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(Received: 21 March 2016; accepted: 30 August 2016)

*Francisella tularensis* is a highly virulent intracellular pathogen with the capacity to infect a variety of hosts including humans. One of the most important proteins involved in *F. tularensis* virulence and pathogenesis is the protein DsbA. This protein is annotated as a lipoprotein with disulfide oxidoreductase/isomerase activity. Therefore, its interactions with different substrates, including probable virulence factors, to assist in their proper folding are anticipated. We aimed to use the immunopurification approach to find DsbA (gene locus FTS\_1067) interacting partners in *F. tularensis* subsp. *holarctica* strain FSC200 and compare the identified substrates with proteins which were found in our previous comparative proteome analysis. As a result of our work two FTS\_1067 substrates, D-alanyl-D-alanine carboxypeptidase family protein and HlyD family secretion protein, were identified. Bacterial two-hybrid systems were further used to test their relevance in confirming FTS\_1067 protein interactions.

**Keywords:** bacterial two-hybrid assay, *Francisella tularensis*, FTS\_1067 protein, immunopurification

## Introduction

*Francisella tularensis* is a Gram negative bacterium that causes the disease tularemia. One of the important *F. tularensis* proteins critical for its virulence is the conserved lipoprotein DsbA (gene locus FTT\_1103 in *F. tularensis* subsp. *tularensis* strain SchuS4, FTS\_1067 in *F. tularensis* subsp. *holarctica* strain FSC200, FTL\_1096 in *F. tularensis* subsp. *holarctica* strain LVS, also referred to as FipB) with proved oxidoreductase and isomerase activities [1–3]. It was shown that the deletion of the gene encoding DsbA led to the high attenuation *in vivo* associated with the ability to induce host-protective immunity [4, 5]. Recently, it was presumed that the DsbA is not a virulence factor by itself but that its substrates, whose correct

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folding and topology are dependent on the DsbA oxidoreductase and/or isomerase activities, are real virulence factors [2]. Unlike other bacterial periplasmic localized DsbA, *F. tularensis* DsbA protein is outer membrane bounded and lipidated [6, 7]. Furthermore, *F. tularensis* DsbA is unusual when compared to other DsbA orthologs; in that it contains N-terminal Forskolin-binding protein-N (FKBP-N) dimerization domain and C-terminal DsbA domain [8].

To find new FTS\_1067 interacting partners/substrates in virulent *F. tularensis* subsp. *holarctica* strain FSC200, we decided to use immunoprecipitation method. The FTS\_1067 protein was FLAG-tag labeled and together with its binding partners was purified on the basis of its interaction to covalently bound mouse IgG monoclonal antibody. Protein–protein interactions identified by mass spectrometry analysis were further studied by means of bacterial two-hybrid (B2H) systems. B2H systems were developed as a faster alternative to yeast two hybrid assays. The most of them utilize *Escherichia coli* transcriptional machinery to detect protein–protein interactions [9]. In these systems, proteins fused to DNA-binding protein [zinc-finger DNA binding domain (Zif) from the murine Zif268 protein] interact with proteins fused to  $\omega$  subunit of *E. coli* RNA polymerase (RNAP), which facilitates transcriptional activation of a Zif-dependent *lacZ* reporter gene. An alternative, the bacterial adenylate cyclase two-hybrid (BACTH) system is based on the reconstitution of adenylate cyclase activity in *E. coli cya*<sup>−</sup> strain [10–12]. The predicted interacting partners (bait and preys) are fused to the T25 and T18 fragments of adenylate cyclase catalytic domain. If there is an interaction between these two tested proteins, both adenylate cyclase fragments reconstitute the enzyme which leads to cyclic adenosine monophosphate (cAMP) synthesis. The cAMP binds to the catabolite activator protein and this complex turns on the expression of several genes including genes of the *mal* operon. The bacteria then utilize maltose as a unique source of carbon and can be distinguished on an indicator or selective media.

The goal of present study was to confirm the proposed FTS\_1067 protein–protein interactions reported previously [5] using either immunopurification of FLAG-tagged FTS\_1067 protein from *trans*-complemented *F. tularensis* FSC200/ $\Delta$ FTS\_1067 strain or the B2H systems and also to confirm whether these systems are suitable for this purpose.

## Materials and Methods

### *Bacterial strains, plasmids, and growth conditions*

The bacterial strains and plasmids used in this study are listed in Table I. *F. tularensis* FSC200 and the derivative strains were routinely cultured at 37 °C

**Table I.** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source and/or reference
<b><i>F. tularensis</i> strains</b>		
<i>F. tularensis</i> subsp. <i>holarctica</i> strain FSC200	Wild-type <i>F. tularensis</i> subsp. <i>holarctica</i> , clinical isolate	<i>Francisella</i> strain collection (FSC) of the Swedish Defense Research Agency, Umeå, Sweden
FSC200/ΔFTS_1067	FSC200/Δ <i>fts_1067</i>	[5]
cFSC200/FLAG	FSC200/ΔFTS_1067 + pKK289-FTS_1067FLAG	This study
<b><i>E. coli</i> strains</b>		
XL1-Blue	<i>endA1 gyrA96(nal<sup>R</sup>) thi-1 recA1 relA1 lac glnV44 F' [::Tn10 proAB<sup>+</sup> lac<sup>I</sup> Δ(lacZ) M15] hsdR17(r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>)</i>	Stratagene
DHM1	F = <i>cya-854 recA1 endA1 gyrA96 (Nal<sup>r</sup>) thi-1 hsdR17 spoT1 rfbD1 glnV44(AS)</i>	Euromedex
KDZif1ΔZ	<i>araD(gpt-lac)5, rpsL (str<sup>R</sup>), ΔspoS3::cat (Cam<sup>R</sup>) [F' lac<sup>I</sup> (Z321[-61] lacZYA*) Kan<sup>R</sup>]</i>	[13]
<b>Plasmids</b>		
pCR4-TOPO	TOPO cloning vector Amp <sup>R</sup> , Km <sup>R</sup>	Invitrogen
pKK289 <i>gfp</i>	<i>Ft</i> ori, p15a ori, Km <sup>R</sup> , <i>groES</i> promoter	[14]
pKK289-FTS_1067FLAG	pKK289 with <i>fts_1067</i> gene fused with FLAG tag	This study
pACTR-Ap-Zif	B2H system plasmid, Tet <sup>R</sup>	[13]
pBRGP-ω	B2H system plasmid, Ap <sup>R</sup>	[13]
pACTR-Ap-MglA-Zif	pACTR-Ap-Zif with full-length MglA	[15]
pBRGP-SspA-ω	pBRGP-ω with full-length SspA	[15]
pACTR-Ap-Zif/FTS_0036	pACTR-Ap-Zif with FTS_0036	This study
pACTR-Ap-Zif/FTS_1034	pACTR-Ap-Zif with FTS_1034 (without signal sequence)	This study
pBRGP-ω/FTS_1067	pBRGP-ω with FTS_1067 (without signal sequence)	This study
pKT25	BACTH system plasmid carrying T25 fragment downstream MCS	Euromedex
pUT18C	BACTH system plasmid carrying T18 fragment downstream MCS	Euromedex
pKT25/FTS_0036	pKT25 with intact FTS_0036	This study
pUT18C/FTS_0036	pUT18C with intact FTS_0036	This study
pKT25/FTS_1034	pKT25 with intact FTS_1034	This study
pUT18C/FTS_1034	pUT18C with intact FTS_1034	This study
pKT25/FTS_1067	pKT25 with intact FTS_1067	This study
pUT18C/FTS_1067	pUT18C with intact FTS_1067	This study

Note: MCS = multiple cloning site.

on McLeod agar enriched for bovine haemoglobin (BD Diagnostics, NJ, USA) and IsoVitaleX (BD Diagnostics, NJ, USA). *E. coli* strains were grown in Luria–Bertani (LB) media or on LB agar plates. When required, kanamycin, 20  $\mu\text{g mL}^{-1}$  (*F. tularensis*) or 50  $\mu\text{g mL}^{-1}$  (*E. coli*); carbenicillin, 100  $\mu\text{g mL}^{-1}$ ; tetracycline, 10  $\mu\text{g mL}^{-1}$ ; ampicillin, 100  $\mu\text{g mL}^{-1}$  or 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were used.

### *Complementation in trans*

The gene coding for FTS<sub>1067</sub> was PCR-amplified using primers FTS<sub>1067</sub>-Fw and FTS<sub>1067</sub>-Rev (Supplementary Table I) and *F. tularensis* subsp. *holarctica* strain FSC200 chromosomal DNA as the template. The reverse primer was designed to introduce DNA sequence coding for FLAG tag (DYKDDDDK) downstream the gene. The PCR fragment was cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, USA) and sequenced (Generi-Biotech, Hradec Kralove, Czech Republic). Using restriction sites for *Nde*I and *Sac*I enzymes, the fragment was ligated to pKK289KmGFP shuttle vector instead of *gfp* gene. The final construct was introduced to *F. tularensis* deletion mutant strain FSC200/ $\Delta$ FTS<sub>1067</sub> [5] by electroporation [16].

### *Purification of FTS<sub>1067</sub> interacting partners*

The complemented strain cFSC200/FLAG was grown on McLeod agar plates supplemented with kanamycin for 24–48 h. Then the bacteria were harvested and washed twice with phosphate buffered saline and finally re-suspended in lysis buffer [50 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; protease inhibitor cocktail Complete EDTA-free (Roche, Basel, Switzerland); and Benzonase Nuclease (Sigma-Aldrich, St. Louis, USA)]. The bacterial lysate was prepared using French Press Pressure Cell by three passages at 16,000 psi and the intact bacteria and cell debris were removed by centrifugation (6,500 rpm, 30 min, 4 °C). Anti-FLAG M2 Affinity Gel (Sigma-Aldrich, St. Louis, USA) was used for immunoprecipitation of the FTS<sub>1067</sub> together with its interacting partners from the cell lysate. The 3  $\times$  FLAG peptide (Sigma-Aldrich, St. Louis, USA) which competes with FLAG-tagged protein was used for elution. As a negative control the same procedure was applied using the deletion mutant strain FSC200/ $\Delta$ FTS<sub>1067</sub>. The presence of FTS<sub>1067</sub> protein in the cell lysate of complemented strain was confirmed by immunodetection using either monoclonal antibody raised against FLAG tag epitope (Anti-FLAG M2 monoclonal antibody, Sigma-Aldrich, St. Louis, USA) or polyclonal antibody against FTS<sub>1067</sub> protein (Apronex, Vestec, Czech Republic).

### Identification of proteins

The liquid chromatography-matrix assisted laser desorption/ionization (LC-MALDI) workflow was applied for identification of FTS\_1067 interacting partners. First, the excess of 3 × FLAG peptide was removed by methanol/chloroform extraction [17] and the sample was resuspended in 0.1% RapiGest ST Surfactant (Waters, Milford, USA). Following reduction and alkylation, the proteins were digested by trypsin (Promega, Madison, USA). Peptides were separated on an Atlantis dC18 3 µm, 0.075 × 150-mm column and fractions were collected on MALDI target plate using the Probot (Dionex, Sunnyvale, USA). The mass spectrometric analysis was performed on a 4800 MALDI TOF/TOF Analyzer (AB Sciex, Framingham, USA). The mass spectra of peptides were acquired from 800 to 4,000 m/z window in the reflector positive ion mode first, followed by tandem mass spectra acquisition on automatically selected precursor ions. Peptide and protein identifications were performed using ProteinPilot 2.0 software (AB Sciex, Framingham, USA). The data were searched against *F. tularensis* subsp. *holarctica* FSC200 database (UniProt, containing 1,420 entries) with appended common contaminant sequences. The proteins identified by at least one unique high confident peptide, as reported by ProteinPilot software, were considered for further evaluation. The respective protein was claimed as a potential substrate of FTS\_1067 if it was identified in the immunoprecipitate from the complemented strain carrying the FTS\_1067 gene and was undetected in the control sample. The potential substrates of FTS\_1067 identified in both biological replicates are reported.

### B2H and $\beta$ -galactosidase assays

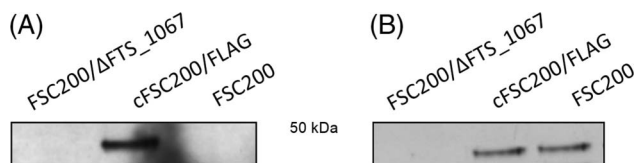
Sequence coding for FTS\_1067 protein without signal sequence was *in frame* cloned to pBRGP- $\omega$  plasmid [13] and sequence coding for FTS\_0036 and FTS\_1034 (without signal sequence) were cloned to pACTR-Ap-Zif plasmid [13]. All plasmid constructs were sequenced. Plasmids and primers used in this assay are listed in Table I and Supplementary Table I, respectively. Plasmids carrying the -Zif and - $\omega$  fusions were cotransformed into *E. coli* KDZif1 $\Delta$ Z [13]. Several clones were inoculated to LB medium containing appropriate antibiotics and grown overnight. Overnight cultures were diluted in fresh LB medium supplemented with antibiotics and 0.5 mM IPTG and grown to OD<sub>600</sub> 0.5–0.6 and assayed for  $\beta$ -galactosidase activity [9]. As a negative control, *E. coli* KDZif1 $\Delta$ Z were cotransformed by empty pACTR-Ap-Zif and pBRGP- $\omega$  plasmids; and as a positive control by plasmids pACTR-Ap-MglA-Zif and pBGRP-SspA- $\omega$  [15]. The empty plasmid pBRGP- $\omega$  together with FTS\_1067-Zif was used as another negative control.

### *BACTH assay*

We performed the BACTH assay according to Battesti and Bouveret's [18] instructions. Briefly, protein FTS\_1067 and proteins of interest were fused with both, the T18 adenylate cyclase fragment (*in frame* fusions at the C-terminal end of T18 and *in frame* fusions at the N-terminal end of T18) and the T25 adenylate cyclase fragment (*in frame* fusions at the C-terminal end of T25 and *in frame* fusions at the N-terminal end of T25). All plasmid constructs were sequenced. Plasmids and primers used in this assay are listed in Table I and Supplementary Table I, respectively. For each assay, *E. coli* DHM1 chemocompetent cells were transformed by 0.5  $\mu\text{L}$  of the two plasmids carrying the T25 and T18 fusions and spread on LB plates containing 100  $\mu\text{g mL}^{-1}$  ampicillin and 50  $\mu\text{g mL}^{-1}$  kanamycin and incubated for 2 days at 30 °C. Several clones were inoculated to 3 mL of LB containing 100  $\mu\text{g mL}^{-1}$  ampicillin, 50  $\mu\text{g mL}^{-1}$  kanamycin, and 0.5 mM IPTG and incubated overnight at 30 °C. The next day, 2  $\mu\text{L}$  of each culture were dropped on MacConkey/maltose plates containing appropriate antibiotics and IPTG; and LB plates supplemented with appropriate antibiotics, IPTG and X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside, 40  $\mu\text{g mL}^{-1}$ ). The plates were then incubated several days at 30 °C until a red/blue coloration appears. Two empty pUT18 and pKNT25 plasmids were used as a negative control, plasmids pKT25-*zip* and pUT18C-*zip* carrying leucine zipper of GCN4 [10] fused *in frame* to the T25 and T18 were used as a positive control. As another negative control, empty pUT18C and pKT25 plasmids together with pKT25 and pUT18C plasmids carrying FTS\_1067 were used. As another positive control pUT18C and pKT25 plasmids carrying *F. tularensis* *iglA*, and *iglB* genes, respectively, were used. IglA–IglB interaction has been previously demonstrated in *F. tularensis* [19].

## Results

In order to purify and identify the candidate binding partners/substrates of FTS\_1067 protein, we *trans*-complemented the FTS\_1067 deletion mutant strain with the shuttle vector pKK289Km carrying the intact gene coding for FTS\_1067 fused with the sequence encoding FLAG tag epitope. The presence of FTS\_1067 protein in the cell lysate of complemented strain was confirmed by immunodetection using either Anti-FLAG M2 monoclonal antibody or polyclonal antibody raised against FTS\_1067 protein (Figure 1). The theoretical molecular weight of FTS\_1067 is 39 kDa, but it is known that the protein is post-translationally modified by glycosylation [20, 21], which increases its molecular weight. We detected the protein band migrating around 50 kDa by both antibodies, proving



**Figure 1.** Detection of FTS<sub>1067</sub> protein in *trans*-complemented strain. The bacterial cell lysates prepared from the deletion mutant strain FSC200/ΔFTS<sub>1067</sub>, the *trans*-complemented strain cFSC200/FLAG, and the FSC200 were separated on SDS-PAGE, then transferred to PVDF membrane by using semi-dry Western blotting procedure, and proteins were visualized using Anti-FLAG M2 monoclonal antibody (A) and polyclonal antibody against FTS<sub>1067</sub> protein (B)

that the protein FTS<sub>1067</sub> is produced in the complemented strain. The amount of FTS<sub>1067</sub> protein in *trans*-complemented strain is comparable to that of wild-type strain, which was documented earlier [3].

Previously, it was demonstrated that the deletion of gene coding for the FTT<sub>1103</sub> or FTS<sub>1067</sub> resulted in the attenuation in mouse model of infection and in the defect in proliferation *in vitro* [4, 5]. Therefore, the effect of complementation was also studied by *in vitro* proliferation of the complemented strain in murine bone marrow-derived macrophages. The proliferation was compared to those of the wild-type FSC200 and the deletion mutant FSC200/ΔFTS<sub>1067</sub> strains and showed that the complemented strain is able to proliferate in this cell line comparably to the wild-type strain FSC200 [3], which suggests the production of fully functional FTS<sub>1067</sub> protein in the complemented strain.

In our study, we tried to purify and identify FTS<sub>1067</sub> substrates by immunoprecipitation. Following immunoprecipitation and elution the complex with 3 × FLAG peptide, the proteins were digested and analyzed by LC-MALDI. Two biological replicates were prepared and only potential substrates of FTS<sub>1067</sub> identified in both replicates on at least two peptides were accepted and are summarized in Table II. These proteins are HlyD family secretion protein FTS<sub>0036</sub> and D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein) family protein FTS<sub>1034</sub> (DacD). As expected, protein FTS<sub>1067</sub> was identified only in the precipitate from the complemented strain and not detected in the control sample (Supplementary Table II). The other identified proteins that did not fulfil the above-mentioned criteria were omitted.

Recently, it was shown that both FTS<sub>1067</sub> and FTT<sub>1103</sub> proteins need to be reduced to act as an isomerase or oxidoreductase [1, 3]. Due to this fact, we decided to further validate the observed protein-protein interactions by using an independent approach, the B2H systems where the interactions take place in reducing cytoplasm.

**Table II.** Substrates of FTS\_1067 identified from immunoprecipitation experiments

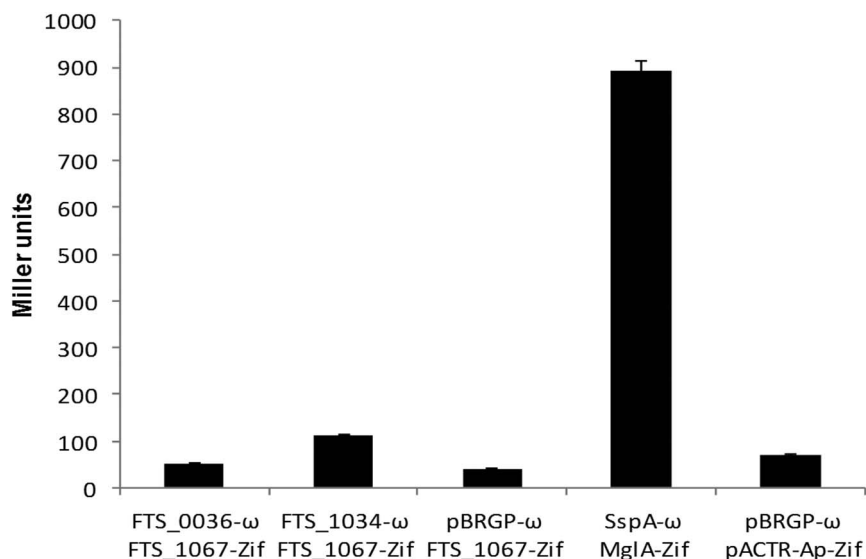
	UniProt ID	Protein name	ORF name	Peptide ID confidence (%)	Peptide sequence
Biological replicate I	K0E1G6	HlyD family membrane fusion protein	FTS_0036	99	ATTNGYISNFILEEGDFIK
				99	DAYLYADFTR
				99	GITRPEYNNFSALYNIER
				99	SLISSESQFIR
	K0E6Y1	D-alanyl-D-alanine carboxypeptidase	FTS_1034	99	TGHTDAAGYCLVSSVK
Biological replicate II	K0E1G6	HlyD family membrane fusion protein	FTS_0036	98	GDNFVLQNNR
				99	GITRPEYNNFSALYNIER
				98	SLISSESQFIR
				99	GDNFVLQNNR
	K0E6Y1	D-alanyl-D-alanine carboxypeptidase	FTS_1034	99	SYIYNFPEAYK
				99	TGHTDAAGYCLVSSVK

*Note:* ORF = open reading frame; ID = identification. The peptide sequences and peptide identification confidence identified for each protein are shown.

First, we employed the *E. coli* B2H system, where the amount of produced  $\beta$ -galactosidase is proportional to the strength of protein–protein interaction [9]. The protein FTS\_1067 was fused to the N-terminus of  $\omega$  subunit of RNAP and proteins of interest were fused to the N-terminus of Zif. *In silico* analyses performed with tested proteins showed that FTS\_1067 and FTS\_1034 contain N-terminal signal sequence (SignalP 4.0) [22]. To prevent secretion of FTS\_1067 and FTS\_1034 fusion proteins outside the cytoplasm, the signal sequences of these proteins were omitted. Unfortunately, the measurements of the  $\beta$ -galactosidase activity (Figure 2) did not prove the interaction of FTS\_1067 with either of the two tested proteins. Similarly, all other proteins identified in the comparative proteome study [5] yielded negative results when assayed for the interactions as well (Pavkova and Schmidt, personal communications).

As a second option of the B2H system, we chose the BACTH assay [10]. The proteins of interest were fused to both T18 and T25 adenylate cyclase domains, and similarly, the FTS\_1067 was fused to either T18 or T25 adenylate cyclase domains. Both genetic fusions at either the C- or N-termini of respective adenylate cyclase domains were constructed in order to avoid a possible shift of



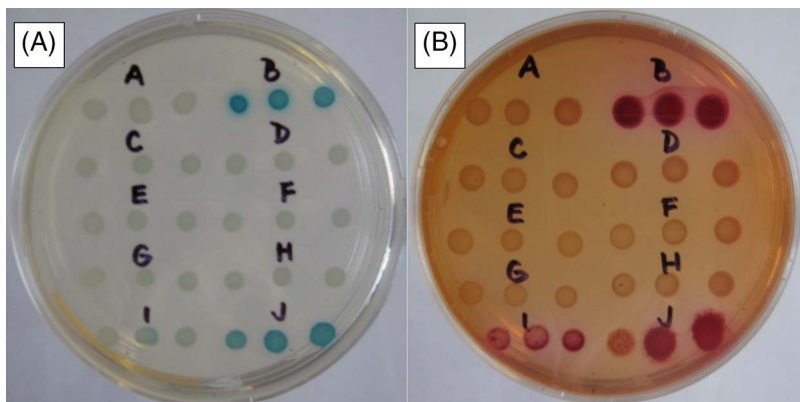


**Figure 2.** Bacterial two-hybrid analysis. Reporter strain *E. coli* KDZif1 $\Delta$ Z carrying plasmids with -Zif and - $\omega$  fusions was subjected to measurements of  $\beta$ -galactosidase activity. As a positive control, MglA-Zif and SspA- $\omega$  fusions were used. As a negative control, two empty pACTR-Ap-Zif and pBRGP- $\omega$  plasmids were used. The mean value for  $\beta$ -galactosidase activity (in Miller units)  $\pm$  standard deviation from two independent experiments including two independent transformants is shown

monitored fusion proteins into periplasm, where the ATP is missing. All combinations of plasmids carrying the T18 and the T25 fusions were tested and gave us the negative results. The results shown in Figure 3 are from experiment where all tested proteins were fused to the C-terminus of either T18 or T25 adenylate cyclase domains. Only the test of the interaction between IglA and IglB proteins, which has been previously described [19], provided a positive signal (Figure 3 – I and J).

## Discussion

Protein DsbA belongs to the most studied *F. tularensis* proteins because of its key role in *Francisella* virulence [1–5, 21, 23]. In fact, the role of DsbA protein in virulence is indirect and depends on its correct oxidoreductase [1, 5, 23] and recently confirmed isomerase activities [1–3]. Hence, it is highly desirable to identify the FTS\_1067 substrates that might be responsible for the FTS\_1067 contribution to *F. tularensis* subsp. *holarctica* strain FCS200 pathogenesis.



**Figure 3.** BACTH assay. Test of protein–protein interactions between FTS<sub>1067</sub> and proteins of interest. 2  $\mu$ L of *E. coli* DHM1 bacterial cultures carrying pKT25 and pUT18C derivatives were dropped onto indicator plates. Three colonies were chosen for each tested pair. (A) LB plate supplemented with kanamycin, ampicillin, IPTG, and X-Gal. (B) MacConkey plate supplemented with kanamycin, ampicillin, maltose, and IPTG. A-BACTH negative control, B-BACTH positive control, C-FTS<sub>0036</sub>pUT18C + FTS<sub>1067</sub>pKT25, D-FTS<sub>0036</sub>pKT25 + FTS<sub>1067</sub>pUT18C, E-FTS<sub>1034</sub>pUT18C + FTS<sub>1067</sub>pKT25, F-FTS<sub>1034</sub>pKT25 + FTS<sub>1067</sub>pUT18C, G-pUT18C + FTS<sub>1067</sub>pKT25, H-pKT25 + FTS<sub>1067</sub>pUT18C, I-IglApUT18C + IglBpKT25, and J-IglApKT25 + IglBpUT18C

The first protein revealed by our immunoproteomic approach was D-alanyl-D-alanine carboxypeptidase family protein FTS<sub>1034</sub>. The same protein was identified also in our proteome study reported previously [5], which suggest that this is indeed a substrate of FTS<sub>1067</sub>. The D-alanyl-D-alanine carboxypeptidase family protein FTS<sub>1034</sub> belongs to the protein family of penicillin-binding proteins. The members of this family catalyze final transpeptidase reaction during the bacterial wall biosynthesis. The homolog of this protein in *Brucella abortus* is a known virulence factor [24]. Very recently the FTS<sub>1029</sub> protein, the homolog of FTS<sub>1034</sub> in SchuS4 strain, was described as an uncharacterized virulence factor candidate [25]. The amino acid sequence of the FTS<sub>1034</sub> contains 3 cysteine residues; one of them seems to be involved in an inter-molecular disulfide bond formation [26–28].

The second identified protein, HlyD family secretion protein FTS<sub>0036</sub>, belongs to the family of membrane fusion proteins, which are responsible for the export of many compounds across the membrane and is essential for the HlyA secretion in *E. coli* [29]. Its protein sequence contains four cysteine residues; thus it might be a substrate for FTS<sub>1067</sub> protein although the prediction (DiANNA 1.1 web server) suggests that all residues are in the form of free-cysteines.

We tried to verify these two proteins as substrates of FTS\_1067 using B2H assays and thus found out their applicability in confirming non-cytoplasmic protein interactions. But, both tested B2H systems gave us negative results. One should argue that the cytoplasm, where interactions in B2H assays take place is reducing environment and that the FTS\_1067 must be in oxidized state to act as an oxidoreductase or isomerase. But as was mentioned above, the results of our group [3] and the results by Qin et al. [1] indicate that both oxidoreductase and isomerase activities of FTS\_1067 or FTL\_1103 are dependent on their reduced state and thus, the reducing environment in cytoplasm should not be limiting.

Recently, Ren et al. [2] published a study on the identification of FTL\_1096 substrates. They identified more than 50 FTL\_1096 substrates using trapping experiments. Among the identified proteins also, the proteins FTL\_0038 (FTS\_0036), FTL\_1060 (FTS\_1034), FTL\_1306 (FTS\_1279), FTL\_1521 (FTS\_1485), FTL\_1532 (FTS\_1495), and FTL\_1579 (FTS\_1538) from our study and from the study by Straskova et al. [5] which gave negative results in B2H assays have been documented.

It seems that the FTS\_1067 and its substrates need to be located outside the cytoplasm in order to preserve their interactions and thus they are undetectable for B2H systems. We cannot also rule out the existence of other auxiliary protein which assists the protein FTS\_1067.

To conclude, using the immunoprecipitation approach, we identified D-alanyl-D-alanine carboxypeptidase family protein FTS\_1034 and HlyD family secretion protein FTS\_0036 as substrates of the FTS\_1067 protein in a fully virulent isolate of *F. tularensis* subsp. *holarctica* strain FSC200. Although our attempts to verify these protein-protein interactions using B2H systems failed, the results obtained from independent study performed on vaccine strain [2] strongly support their *in vivo* existence.

### Acknowledgements

The authors would like to thank Åke Forsberg for the gift of pKK289KmGFP plasmid and Jeanette E. Bröms and Simon L. Dove for providing the bacterial two-hybrid system. This work was supported by Ministry of Defence, Czech Republic Long-term Organization Development Plan 1011 and The Specific Research of Faculty of Military Health Sciences, University of Defence, Czech Republic (Grant No. FVZ0000604).

### Conflict of Interest

The authors have no conflict of interest to declare.

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