

# Comparative Analysis of Proteome Patterns of *Francisella tularensis* Isolates from Patients and the Environment

Murat Kasap<sup>1</sup> · Aynur Karadenizli<sup>2</sup> · Gürler Akpınar<sup>1</sup> · Hüseyin Uzuner<sup>2</sup> ·  
Abula Ayimugu<sup>3</sup> · Kübra Karaosmanoğlu<sup>3</sup> · Doğanhan Kadir Er<sup>2</sup>

Received: 27 June 2016 / Accepted: 7 December 2016 / Published online: 19 December 2016  
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**Abstract** *Francisella tularensis* is the causative agent of tularemia. Although major contributors and the main mechanism of the virulence are well known, some of the molecular details are still missing. Proteomics studies regarding *F. tularensis* have provided snapshot pictures of the organism grown under different culture conditions to understand the mechanism of virulence. In general, such studies were carried out with standard strains e.g., LVS and did not involve comparisons of *F. tularensis* isolates from either clinical or environmental sources. In this study, we performed two-dimensional gel electrophoresis (2DE)-based proteomic analysis and compared the protein profiles of the *F. tularensis* subsp. *holarctica* strains isolated from the clinical and the environmental samples. Regulations were detected in 14 spots when twofold regulation criteria were applied. The regulated protein spots were subjected to MALDI-TOF/TOF analysis and identified. Classification of the identified proteins based on metabolic functions revealed that the translation machinery was the most varying metabolic processes among the isolates. Using normalized protein spot intensities, PCA analysis was also performed. The results indicated that the strain isolated from water source was different than the strains isolated from the patients. Most interestingly, the isolates were strikingly distinguishable from the standard NCTC 10857 strain.

## Introduction

*Francisella tularensis* is the causative agent of tularemia [3, 21]. If not treated, the pneumonic form of tularemia is lethal. Although the organism cannot be transmitted from human to human, it can be transmitted through water resources, infected animals, or vectors e.g., ticks and mosquitoes [4]. That is why the tularemia cases have been increasingly observed over the last decade mostly in developing countries, like Turkey [6]. The transmission of *F. tularensis* is facilitated by the fact that the organism can survive outside of a host for weeks and has been detected in water, grassland, and haystacks [2, 5, 11]. The facultative nature of *F. tularensis* also contributes to its transmission process because the organism can infect the most cell types.

Although major contributors and the main mechanism of the virulence are well known, the molecular details are still missing. In short, after the intake of *F. tularensis*, the organism infects the macrophages via phagocytosis forming an organelle-like structure called phagosome [24]. The phagosome is then degraded by *F. tularensis* via production of different hemolytic agents. The involvement of proteins to *F. tularensis* virulence is known [35]. For instance, a 23 kDa protein known as IgIC is required for phagosomal breakout and intracellular replication. Along with IgIC, there are several other proteins that may play important roles in the replicative process by hindering the immune response of the host [14, 16, 31]. Those proteins and the other proteins playing roles in pathogenicity are needed to be discovered.

*Francisella tularensis* has been studied since 1912. Despite of its monomorphic nature, there seems to be a significant degree of plasticity at the genomic level among the isolates [13, 32]. This plasticity is expected to be reflected on to the proteome of *F. tularensis* subspecies, but

✉ Aynur Karadenizli  
aynuryk2010@yahoo.com; aynurk@kocaeli.edu.tr

<sup>1</sup> Medical Biology Department/DEKART Proteomics Laboratory, Kocaeli University Medical Faculty, Umuttepe, Kocaeli 41380, Turkey

<sup>2</sup> Department of Medical Microbiology, Kocaeli University Medical Faculty, Umuttepe, Kocaeli 41380, Turkey

<sup>3</sup> Department of Biomedical Engineering, Kocaeli University Technology Faculty, Kocaeli, Turkey

there are currently no available data to support it. Such plasticity is caused by the presence of IS elements, which provide adaptation of *F. tularensis* to its distinctive ecologies [13]. Current medical practice does not rely on subtypes or subpopulation classification, despite the fact that this information may hold predictive value for clinical outcome. Thus, we believe that combined molecular approaches are needed to truly identify tularemia ecology and understand the very nature of tularemia outbreaks. In this study, we used a 2DE-based proteomics approach to elucidate sample type-dependent changes on protein profiles of *F. tularensis* isolates.

## Materials and Methods

### Sample Collection and Isolation of *Francisella tularensis*

The isolation of *F. tularensis* strains used in this study and their characterization were previously described [28].

### Culturing of *Francisella tularensis*

All strains and a standard *F. tularensis* subsp. holarctica NCTC 10857 were grown in cation-adjusted Mueller–Hinton broth containing 2% IsoVitalax, 0.025 ferric pyrophosphate, and 0.5% glucose at 37 °C with 5% CO<sub>2</sub> for 24 to 48 h.

### Preparation of Protein Extracts

Freshly grown bacteria were washed with ice-cold washing buffer (250 mM Sucrose containing Tris. Cl at pH 7.2) for 3× to remove any residual growth medium. 100 µL per mg of wet weight 2D rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris pH 8.5, and 1× protease inhibitor cocktail) was used to prepare protein extracts. The cells were lysed using a bead beater with 0.2 mm stainless steel beads (Next Advance, USA). Supernatant containing soluble proteins was obtained after centrifugation at 15,000×g at 4 °C for 20 min. and transferred into a low protein binding tube (Eppendorf, USA). Protein concentrations in the samples were measured using Bradford protein dye-binding assay (Bio-Rad, USA).

### Two-Dimensional Gel Electrophoresis

IPG strips (11 cm pH 3–10 NL, Bio-Rad, USA) were passively rehydrated using 2DE Rehydration Buffer containing 300 µg of protein at 22 °C for 16 h. and then were run through a stepwise incremental voltage program [250 V for 20 min (linear), 4000 V for 2.5 h (linear), and

30,000 V/h (rapid)] by using Protean IEF system (Bio-Rad, USA). The strips were then subjected to a two-step equilibration in buffers containing 6 M urea, 2% SDS, 0.375 M Tris.Cl pH 8.8, 20% glycerol, and 2% DTT for the first step and the same buffer without DTT but with iodoacetamide (2.5%) for the second step. Second dimension separation was achieved using TGX precast gels (Bio-Rad, USA) which were stained with Colloidal Coomassie stain (KeraFast, USA) and visualized with VersaDoc4000MP (Bio-Rad, USA) by using Quantity One software (Bio-Rad, USA). Three experimental replicate gels were used for analysis.

### Image Analysis and Statistical Significance

The gel images were analyzed with PDQuest Advanced software (Bio-Rad, USA). The outer edges of the images were identically cropped using the automated crop tool of PDQuest Advance Software (Bio-Rad, USA). Stain speckles were filtered and the standardized areas of interest from all gels were matched and warped; quantity of each spot was normalized by linear regression model. The statistical significance of image analysis was determined by the Student's *t* test (statistical level of  $P < 0.05$  is significant). Gel spots significantly differed in expression (>2-fold) were selected and excised from gels using ExQuest Spot cutter (Bio-Rad, USA) for protein identification. A manual editing tool was used to inspect the determined protein spots detected by the software. The spots were cut using automated spot cutting tool, ExQuest spot cutter (Bio-Rad, USA), and disposed into a 96-well plate for protein identification.

### In-Gel Tryptic Digestion

In-gel tryptic digestion of the proteins was performed using an in-gel digestion kit following the recommended protocol of the manufacturer (Pierce, USA). The selected protein spots were destained/washed with 40% acetonitrile (ACN)/ 50 mM ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>) until the gel pieces become colorless, reduced with 10 mM DTT at 50°C for 30 min, and alkylated in the dark with 50 mM iodoacetamide at room temperature for 30 min. The gel pieces were dehydrated using 200 µl ACN for 15 min with shaking. After dehydration, ACN was removed and gel pieces were dried at room temperature. Tryptic digestion was performed by the addition of 10 ng trypsin in 20 µl 40 mM NH<sub>4</sub>HCO<sub>3</sub> solution for each spot followed by incubation at 37 °C for overnight. After the digestion, the peptides were collected, evaporated in a SpeedVac (Eppendorf, USA), and reconstituted in 10 µl 0.1% trifluoroacetic acid. C18 ZipTip pipette tips (Millipore, USA) were used to desalt/concentrate the peptides according to the manufacturer's instructions. The concentrated peptides were eluted with 0.8 µl matrix

(10 mg/ml *a*-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile and 0.1% trifluoroacetic acid) and directly spotted onto a MALDI sample target plate.

### Protein Identification and Bioinformatic Analysis

Protein identification experiments were performed at Kocaeli University DEKART proteomics laboratory (<http://kabiproteomics.kocaeli.edu.tr/>) by using ABSCIEX MALDI-TOF/TOF 5800 system. Peak data were analyzed with MASCOT using a streamline software, Protein Pilot (ABSCIEX, USA). The parameters for searching were enzyme of trypsin, 1 missed cleavage, fixed modifications of carbamidomethyl (C), variable modifications of oxidation (M), peptide mass tolerance: 50 ppm, fragment mass tolerance:  $\pm 0.2$  Da, peptide charge of 1+, and monoisotopic. Only significant hits as defined by the MASCOT probability analysis ( $P < 0.05$ ) were accepted. Classification of the proteins was performed using a freely available classification system, PANTHER (<http://www.pantherdb.org/>).

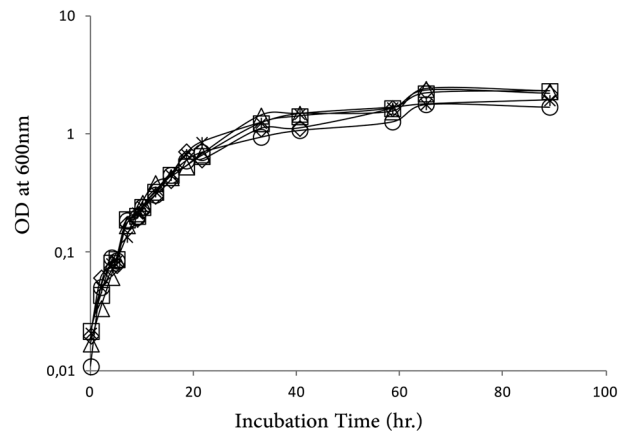
### Principal Component Analysis (PCA)

Numerical analysis for excel (NumXL) was used to perform the PCA analysis. NumXL is a Microsoft Excel add-in software and provides a wide variety of statistical and time series (Spider Financial Inc., USA). Data for each spot intensity were extracted from the PDQuest Advance software and entered into the Excell. The PCA analysis was carried out following the instructions.

### Results

In order to perform comparative proteome analysis, we cultured five different *F. tularensis* isolates and analyzed their growth characteristics. This is a must-do experiment in bacterial comparative proteomic studies since bacteria in different growth phases may display differences in their proteome profiles. The growth curves of each strain indicated that the cells displayed similar growth characteristics and were in the mid-logarithmic phase when their OD values were at  $0.5\text{--}0.6 \pm 0.05$  (Fig. 1). Therefore, the cells were harvested at OD<sub>600</sub> of 0.5 and then used for comparative proteome analysis. For this purpose, soluble protein extracts from each isolate were prepared and subjected to 2DE. Following Colloidal Coomassie staining, well-resolved and reproducible 2DE gel maps were produced as shown in Fig. 2.

An average of  $470 \pm 20$  well-stained protein spots per analytical gel was detected when the gels were subjected to an automated spot detection and analysis. There was an average of 96% match rate with an average correlation coefficient of 0.85 among the gels. By using PDQuest



**Fig. 1** Growth of *F. tularensis* isolates and NCTC strain in cation-adjusted Mueller–Hinton broth containing isoVitalex, ferric pyrophosphate, and glucose medium over a period of 100 h. Symbols represent: open diamond Isolate 1, open square Isolate 2, open triangle Isolate 3, open circle Isolate 4, x NCTC

Advance (Bio-Rad, USA) gel analysis software, changes in spot intensities among the protein spots were compared. In overall, analysis of spot scattering plots, conservation scores, and PCA analysis indicated that the standard strain (NCTC) differed from the other strains noticeably and displayed a visible variation in spot distribution.

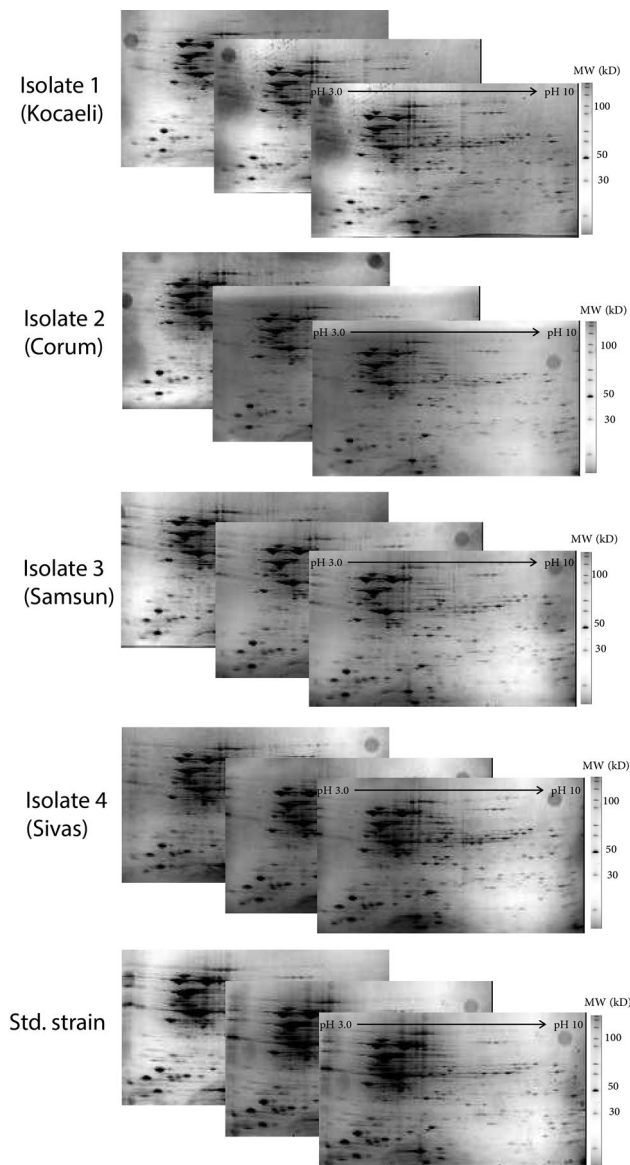
When the PCA analysis was performed, three strains isolated from the same sample type (clinical isolates) were grouped together, although they were from relatively distantly located cities (Kocaeli, Çorum and Sivas) (Fig. 3). On the contrary, the standard strain (NCTC) and the sample isolated from natural spring water stood alone indicating that an overall classification of isolated strains based on PCA analysis of 2DE protein profiles was possible.

When a strict twofold regulation criteria were applied to reveal the presence of regulated protein spots, 14 of them were detected (Fig. 4). To further characterize the regulated protein spots, the spots were cut by an automated spot cutter, subjected to in-gel tryptic digestion, and identified by MALDI-TOF/TOF analysis (Table 1). The identified proteins were grouped based on their molecular function and their involvement in biological processes (Fig. 5).

The majority of the identified proteins play roles in translation machinery indicating that the cells were actively growing. There were other proteins that play roles in various metabolic events including energy metabolism, protein folding, and DNA repair.

### Discussion

*Francisella tularensis* is one of the most life threatening organisms [1]. As few as ten bacteria are sufficient to cause deadly infections [17]. Understanding pathogenesis of



**Fig. 2** The 2DE gel images were used for analysis of *F. tularensis* isolates and the NCTC strain. IPG strips (11 cm, pH 3–10NL) were used for the first dimension and TGX precast gels were used for the second dimension separations. For analysis of the images, PDQuest Advance software was used. Spots that were regulated were cut by ExQuest Spot cutter and subjected to MALDI-TOF/TOF analysis

*F. tularensis* requires comprehensive knowledge of the proteins expressed by the pathogens as well as the host during infection [34, 36]. Similar to other pathogens, *F. tularensis* may use various invasion strategies and some of which may depend on the changes on protein expression and post translational modifications [25, 26]. Since the year 2000, there have been serious efforts placed on understanding the proteome of *F. tularensis* to achieve two different aspects. The first aspect of the studies focused on the creation of a *F. tularensis* 2DE database [8, 10, 19, 20, 27, 33]. Such a database is helpful in

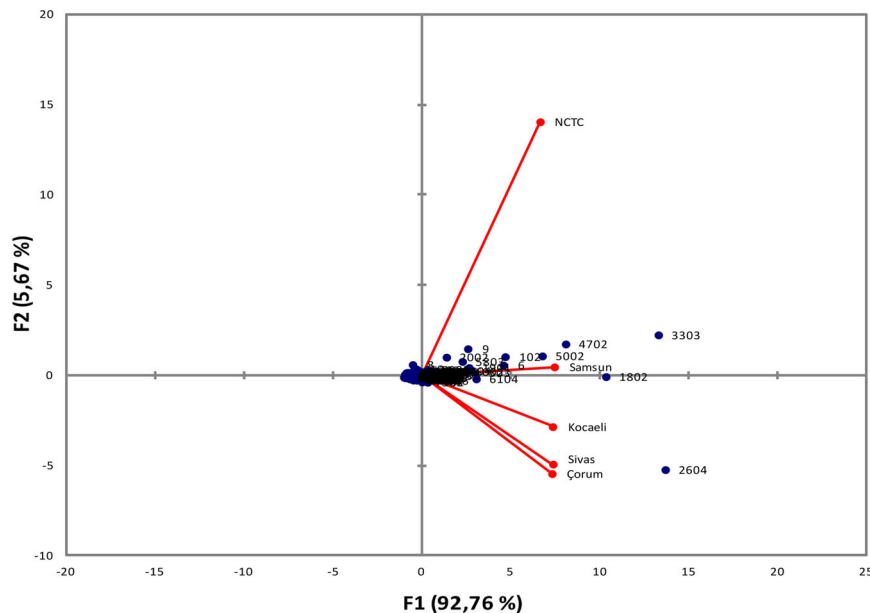
obtaining a catalogue of proteins expressed by *F. tularensis* and could be used at early detection, identification, typing, and diagnosis of tularemia. A 2DE database containing information about *F. tularensis* proteome was created and is now available on the net (<http://web.mpiib-berlin.mpg.de/cgi-bin/pdbs/2d-page/extern/index.cgi>). The information stored in the database, however, is limited and cannot be used beyond descriptive purposes. The second group of studies identified proteins whose expressions are related to pathogenicity [9, 12, 15, 22, 29, 30, 32]. Those studies cultivated *F. tularensis* under the conditions that mimicked the hostile intracellular milieu and monitored the changes in protein profiles in comparison to standard culture conditions. A list of proteins whose expression levels are associated with stress conditions such as heat, acid stress, and nutritional defects were generated. The results demonstrated that each stress condition induced expression of its own distinctive set of proteins [10, 18].

Despite the recent progress, much remains to be understood about the molecular basis of *F. tularensis* pathogenicity in order to promote development of therapeutics, diagnostics, and prophylactic tools against tularemia. Successful new strategies in understanding the molecular mechanisms of virulence should include the work carried out not only with the virulent strains but also with the strains isolated from the environment. In this study, we performed a comparative proteomic study using three clinical and an environmental isolates of *F. tularensis*. The proteome profile of each strain was elucidated and compared. The comparisons demonstrated an extremely similar protein expression patterns. This extreme similarity in protein expression patterns might be the result of the organisms adaptation to in vitro culture conditions. It appears that *F. tularensis* can rapidly modify its protein expression pattern and adapt to its environment. To point out the nature of this problem, a group of scientists developed a novel immunomagnetic isolation-based experimental approach to isolate *F. tularensis* from its natural environment [34]. The authors claimed that a far different proteome was expressed by the pathogen in vivo than in vitro, and the mimicking studies to stimulate host environment were not realistic [7]. Despite of this fact, researchers still benefit from the data generated by the proteomic studies of *F. tularensis* grown in in vitro culture conditions. The areas benefit from these studies include vaccine development, visualization of the metabolic networks grown under different culture conditions, improvement of genome annotations that is made based on bioinformatics predictions, discovery of hypothetical proteins, and validation of operons.

In here, we wished to discuss the importance of our data in two different ways. One of the ways was to identify the differentially expressed proteins among the clinical and



**Biplot (axes F1 and F2: 98,43 %)**

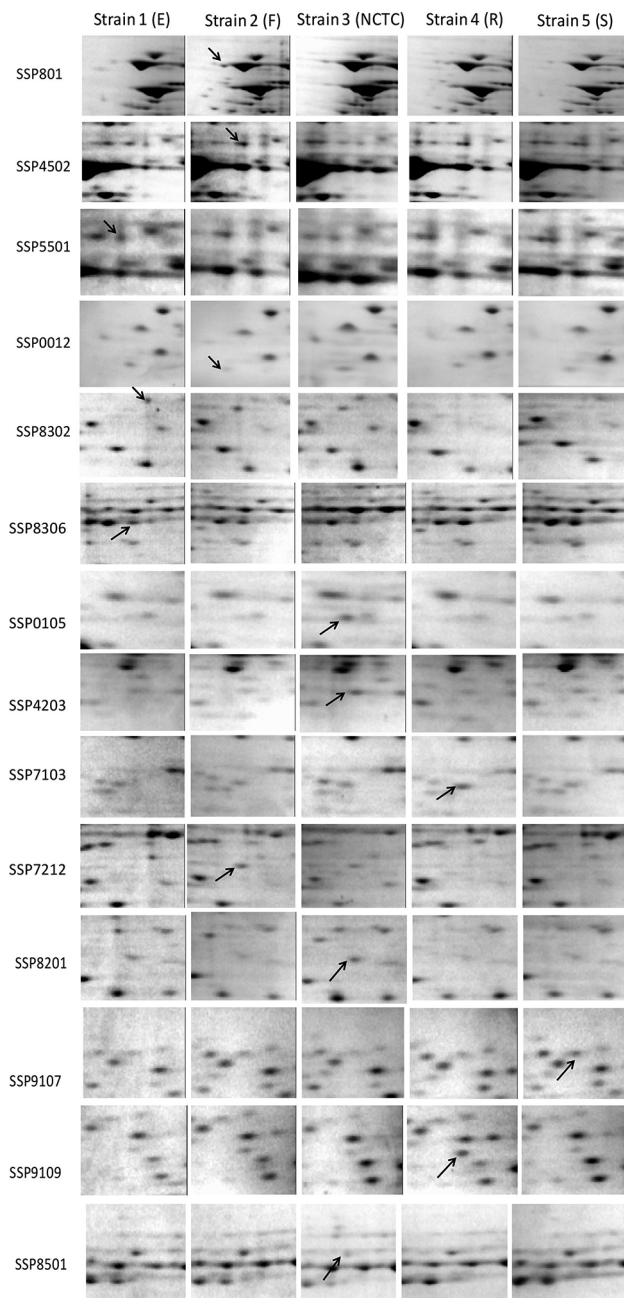


**Fig. 3** The map of Turkey showing the cities where *F. tularensis* isolates were obtained. The below *diagram* was generated from the PCA analysis to show the similarities among the isolates. Data for

each spot intensity were extracted from the PDQuest Advance software and used as an input for NumXL, an add-in PCA analysis software

environmental isolates and the standard strain. Such comparisons yielded identification of 14 differentially expressed proteins when 2-fold regulation criteria were applied. There were 3 proteins whose expression levels were high and a protein whose expression level was low in the standard strain in comparison to the clinical and the environmental isolates. Among those regulated proteins, there was a hypothetical membrane protein that was not previously known to be expressed. The detection of expression

of hypothetical membrane proteins is especially important since almost 30% of the annotated *F. tularensis* proteins are hypothetical; their functions are unknown and waiting to be explored. In addition, seryl-tRNA synthetase appeared to be downregulated in the standard strain in comparison to the environmental and clinical isolates, and the reason behind this observation was not clear. *F. tularensis* isolated from the environmental sample did not show much variation in its protein expression levels. There



**Fig. 4** Close-up images of the 2DE gels for visual inspection of the regulated protein spots. SSP numbers were assigned to the spots by PDQuest Advance Software. The *arrows* point to the spot of interest that was cut from the corresponding gels and were identified by MALDI-TOF/TOF analysis

was an increase observed in the expression level of beta-lactamase in comparison to the other isolates. Whether this increase in beta-lactamase level causes an additional antibiotic resistance to the isolate has to be tested.

The regulated proteins in this study involved in translation and other energy metabolisms. Yet, the strains had similar growth rates contradicting the expectation that cells displaying changes in translation machinery should have

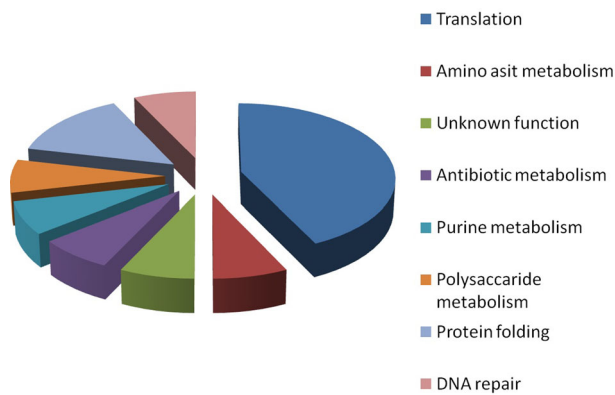
similar protein synthesis and replication rate and thus growth pattern. However, it may be too simplistic to think that changes at the proteome level will readily be reflected on the phenotype. There are post translational modifications (PTMs) that may prevent changes in phenotypic characteristics. Therefore, in our case, it may be possible that PTMs that are not the focus of this study prevented immediate reflection of the changes occurring at the proteome level to the growth of *F. tularensis* isolates.

The present study also demonstrated that the soluble proteome of *F. tularensis* did not reveal major differences in protein expression patterns among the isolates irrespective of their origin of isolation. This is especially true when two- or morefold of regulation criteria was applied to analyze the 2DE gels. However, this observation did not necessarily rule out the fact there were minor changes in protein expression patterns among the isolates and those changes might be useful to elucidate the differences. In order to test this idea, we registered intensities of 169 protein spots and used them in PCA analysis for comparative purposes. PCA involves a mathematical procedure and can be used as an exploratory tool to identify trends in a multidimensional dataset and to find samples that tend to vary in their trend. There are several publications which used the PCA approach to simplify their 2DE gel data and create relevant groups from their study sets [23]. We also used our dataset that we created using spot intensities for PCA analysis. The results demonstrated the presence of a clear clustering for the clinical isolates. The environmental isolate, although formed a sister cluster to the clinical isolates was not included within the group. In addition, the standard strain, NCTC, was located distantly from the clinical isolates and the environmental isolate indicating that in overall there was a distinctive proteome profile for this strain. Such a clear separation of this strain might be caused by the adaptive response of it to in vitro conditions during so many generations of cultivation. Another important point to make from the PCA analysis was that the clustering of the isolates was independent of their isolation locations. For instance, although distantly located cities, the proteome profile of the isolate from the city of Sivas closely resembled to the proteome profile of the isolate from the city of Corum indicating that proteome analysis may not be used to trace the origin of epidemics.

Several conclusions can be drawn from this study: (1) Analysis of soluble proteome of *F. tularensis* did not yield significant differences under in vitro culture conditions suggesting that insoluble proteome analysis should rather be performed. (2) Studies dealing with the changes in proteome of *F. tularensis* using in vivo conditions might generate more realistic data as demonstrated before [34]. (3) Clustering of *F. tularensis* based on the changes in intensities of protein spots was possible. In overall,

**Table 1** MALDI-TOF/TOF analysis results used for identification of the proteins cut from 2D gels

SSP#	Swiss-prot AC no.	Best protein Acc.	Best protein mass	Best protein score	Expectation score	Matches	Calc. pI	Seq. cov. (%)	Best protein description	Up-regulated	Down-regulated
5501	Q14ID8	SYE_FRAT1	52,920	443	1.60E-39	21	5.42	31	Glutamyl-tRNA synthetase	In clinical isolate #1	
8302	Q14FQ1	RECA_FRAT1	38,809	84	0.0012	15	5.97	26	Protein recA	In clinical isolate #1	
0801	Q6D0B7	DNAK_ERWCT	68,907	96	7.90E-05	5	4.83	6	Chaperone protein dnaK	In clinical isolate #2	
4502	A0A0P0MMP7	WP_003018129.1	48,817	278	2.90E-24	19	5.29	34	UDP-N-acetyl-D-galactosamine dehydrogenase	In clinical isolate #2	
7212	A7NAH6	GCST_FRATF	39,491	537	6.40E-49	29	5.73	57	Aminomethyltransferase	In clinical isolate #2	
0012	A0A0B3VEP0	WP_003014087.1	13,720	140	1.80E-10	13	8.67	53	Hypothetical membrane protein	In NCTC standard strain	
0105	Q5NI60	WP_003014194.1	20,890	185	5.80E-15	11	4.82	35	Elongation factor P	In clinical isolate #3	
4203	Q14ID1	EFTS_FRAT1	30,968	104	1.30E-05	4	5.57	14	Elongation factor Ts	In clinical isolate #3	
8201	Q5NIJ6	WP_003017322.1	47,368	98	3.00E-06	5	6.12	9	Citrate synthase	In clinical isolate #1	
9109	Q14H66	KAD_FRAT1	24,347	321	2.60E-27	14	7.78	43	Adenylate kinase	In NCTC standard strain	
7103	A0Q5P6	WP_003016758.1	26,848	151	1.50E-11	9	5.87	28	Purine nucleoside phosphorylase	In NCTC standard strain	
8306	A0A0H3TNZ5	WP_003028605.1	46,764	505	5.80E-47	30	6.22	48	Type II citrate synthase	In clinical isolate #1	
8501	Q14GR3	SYS_FRAT1	48,555	510	3.20E-46	18	6.11	38	Seryl-tRNA synthetase		In NCTC standard strain
9107	A0Q6U4	WP_003015518.1	31,945	168	2.90E-13	11	7.66	29	Beta-lactamase	In environmental isolate #4	



**Fig. 5** Functional classification of the identified proteins. The information regarding the function of each protein was extracted from Uniprot database ([www.uniprot.org](http://www.uniprot.org))

although it was carried out with limited number of isolates, this study demonstrated the usefulness of proteomics data for analysis of *F. tularensis* isolates.

Most human tularemia cases in Turkey are water-borne infections and that genetic similarities have been previously found between strains isolated from water and humans in some geographic areas. This probably partly explains why in this country the proteomic profiles of these two kinds of strains are so similar. On the other hand, culturing of both types of strains using the same in vitro experimental medium may have resulted in the attenuation of variations in proteomic expression, which could be much more important in respective natural conditions. However, the clustering of strains from water and those from humans is interesting and warrants further investigation.

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