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Review

Towards proteome database of *Francisella tularensis*

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Abstract

The accessibility of the partial genome sequence of *Francisella tularensis* strain Schu 4 was the starting point for a comprehensive proteome analysis of the intracellular pathogen *F. tularensis*. The main goal of this study is identification of protein candidates of value for the development of diagnostics, therapeutics and vaccines. In this review, the current status of 2-DE *F. tularensis* database building, approaches used for identification of biologically important subsets of *F. tularensis* proteins, and functional and topological assignments of identified proteins using various prediction programs and database homology searches are presented.

Keywords: Review; Proteome databases; *Francisella tularensis*

Contents

1. Introduction

Despite advanced antibiotic therapy, infectious and parasitic diseases still represent the main cause

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of death for mankind. The reality can be even worse due to the increasing number of bacterial strains exhibiting resistance against antibiotics on the one hand and the increased resurgence of seemingly eliminated infections such as tuberculosis or cholera on the other. Thus the only reaction to this new situation should be diligent effort aimed at identifying new molecules with diagnostic and vaccine applications and determining novel targets for more efficient drug therapy. This challenging task is partly alleviated by microbes themselves. Microbes are relatively simple entities that can be grown in chemically defined media providing scientists with sufficient amounts of material for analyses. Furthermore, bacterial genomes are small, hence current technologies, in spite of their inherent limitations, can be successfully applied for the comprehensive scrutiny of microbial genome expression. The restricted scope of prokaryotic genomes is also responsible for the fact that over 90% of the completely sequenced genomes are of microbial origin. According to an updated list (see www.tigr.org) more than 80 microbial genomes have been completely sequenced and, additionally, more than 130 microbial genome-sequencing projects are in progress. The availability of complete genome sequences is prerequisite for more targeted approaches focused on detection of molecules of significance. Until recently, the approaches performed at DNA or RNA level, like comparative genomics or mRNA microarray technology, predominantly throve on genome knowledge. However, both approaches are defective in predicting corresponding protein expression level, its structural modification, cellular localization and possible protein–protein interactions. Therefore, simultaneous analysis of global protein patterns must complement ongoing genetic studies in order to provide more valid information for definitive selection of diagnostic or therapeutic candidates. The rapidly developing procedures combining two-dimensional gel electrophoresis (2-DE) or multidimensional chromatography with mass spectrometry (MS) techniques [1,2], summarized by the term proteomics, are expected to bring major breakthroughs in protein mapping. This review highlights some recent achievements in the application of proteomics for the analysis of bacterial proteomes. Special emphasis is placed on building a proteome database of the bacterial intracellular pathogen *Francisella tularensis.*

1.1. Outline of the current status of bacterial proteome studies

The current studies of bacterial proteomes can be basically divided into two large groups. The first one is concentrated on the building of comprehensive 2-DE protein databases containing the reference 2-DE maps of individual organisms (see http://us.expasy.org/ch2d/2d-index.html). The formation of these databases is now facilitated by concomitant progress in 2-DE, protein staining and, especially, in MS procedure. These databases should serve as the basis to which all other experimental strategies utilizing virulent, mutant strains or strains cultivated under harsh conditions can be then compared. However, all displayed databases exhibit one dominant shortcoming referring to the disproportion between the number of identified genes and the actual number of identified proteins. The reason for this imbalance is the failure of current proteomics technology to provide adequate resolution of proteins with extreme physicochemical properties and, further, the insufficient detection of proteins occurring in cell only in a few copies [3].

Most attention regarding microbial 2-DE database construction is paid to microorganisms that are clinically relevant and/or that have been extensively studied with respect to their genetics and biochemistry for many years. Furthermore, the accessibility of complete genome sequences is also crucial. Two-dimensional protein databases of *Escherichia coli* and *Haemophilus influenzae* belong to the largest ones. As for the former, the recent application of ultra narrow pH gradients enabled display of more than 70% of the entire *E. coli* genome [4]. As for the latter the commonly applied proteomics procedures were extended to the application of several chromatographic steps, including heparin chromatography, chromatofocusing and hydrophobic chromatography. In this way 502 different proteins were identified in what represents about one third of completely sequenced open reading frames (ORF) [5]. The other intensively studied human pathogens are *Mycobacterium tuberculosis* [6] and *Helicobacter pylori* [7].

The alternative approach that should expand the number of identified proteins in 2-DE databases is based on the proteome analysis of purified protein subsets. This is particularly important when membrane and secreted proteins that significantly contribute to the mechanism of pathogenicity have to be studied. Triton X-114 or carbonate extraction followed by solubilization of purified proteins in buffers containing new types of detergents are methods of choice for isolation of integral membrane proteins [8,9]. These protocols were successfully applied in the global analysis of the outer membrane proteome of *Leptospira interrogans* and *Caulobacter crescentus* [10,11]. Bacterial secreted proteins play very diverse roles in host–pathogen interactions. They can reorganize host cytoskeletal structures, modulate cell-signaling pathways and protect engulfed bacteria against toxic molecules produced by infected cell [12]. Additionally, some of the secreted antigens exert strong immunostimulating effects. Weldingh et al. [13] established 2-DE reference maps of *M. tuberculosis* culture filtrate proteins in order to identify candidate antigens for a novel subunit vaccine against tuberculosis. In the case of *Bacillus subtilis* the release of extracellular proteins is associated with the generation of a heat-resistant endospore that enables bacteria to survive under poor nutrient conditions. Examination of the proteome of *B. subtilis* extracellular proteins led to the visualization of more than 100 spots. Of them, over 90% disappeared when mutant strains with deficient Sec protein-secretion pathways were tested [14].

The second group of proteome studies is aimed at identification of proteins whose expression relates to the pathogenicity of wild bacterial strains. The simplest approach exploits the comparative proteome analysis of protein patterns of non-virulent strains and their pathogenic counterparts. This procedure was successfully applied in comparative analysis of protein profiles of avirulent vaccine and virulent laboratory mycobacterial strains where besides the complex cell lysates the cell culture supernatants were also used for strain comparison. The results confirmed the existence of differences in both intensity and mobility between cell proteins extracted from mycobacterial strains differing in pathogenicity [15]. Similarly, unique proteins were found in protein spectra of genetically indistinguishable *Pseudomonas aerigunosa* strains representing initial and chronic isolates from a cystic fibrosis patient [16].

The more sophisticated approach to highlight proteins potentially associated with the progression of bacterial infection is the proteome study of bacteria cultivated under conditions mimicking hostile intracellular milieu or even after their ingestion by phagocytes. Regarding environmental influences the bacterial responses to heat, oxidative, acid stress and to nutrition defects were studied on the proteome level. Each stress condition induced its own distinctive set of genes, which partly overlapped, especially, in the overproduction of chaperonins [17–19]. Likewise, heat-shock proteins were up regulated in *Brucella abortus*, *Leishmania chagasi* and *Legionella pneumophila* growing inside infected host cells [20–22]. As for *L. pneumophila* the induction of cpn60 occurred very early in the course of infection and it was characteristic only for virulent strains.

The highly decisive proteome study from the point of view of vaccine construction is the mapping of bacterial immunorelevant antigens. 2-DE immunoblotting utilizing sera collected from patients as primary antibodies is the predominant technique for monitoring candidate antigens. Haas et al. [23] performed an extensive immunoproteomics study of *H. pylori* infection in which the potential association between specific immune response and manifestation of disease was investigated. They compared the immunoreactivity of serum antibodies collected from patients suffering from active *H. pylori* infection, with a control group with unrelated gastric diseases and, finally, with patients with gastric cancer. The preliminary results confirmed the existence of antigens differently recognized by sera from gastritis and ulcer patients suggesting them as possible indicators of clinical manifestation [23]. Proteomics can also be a powerful method for elucidation of immunodominant T cell antigens. Using two-dimensional liquid phase electrophoresis, Covert et al. [24] separated *M. tuberculosis* culture filtrate and cytosolic proteins into great number of fractions which were tested for T cell stimulating activity via production of interferon γ. Proteins occurring in positively tested fractions were then identified by liquid chromatography-mass spectrometry. Globally, of the 30-mycobacterial proteins with T cell stimulating activity identified, 17 of them were novel antigens.

1.2. Biological and clinical importance of F. tularensis

F. tularensis, the causative agent of tularemia, was first observed in animal tissues by McCoy [25], subsequently isolated by McCoy and Chapin from ground squirrels in Tulare County, California, was considered a ''plague-like disease'', and named *Bacterium tularense* [26]. Shortly thereafter, tularemia was recognized as a rare but potentially severe and fatal illness in humans [27]. The disease is endemically spread over the northern hemisphere. Various small mammals, mainly members of the orders *Rodentia* and *Lagomorpha*, create the principal reservoirs in nature. Transmission to humans and other vertebrates can be mediated by bites of ectoparasites such as ticks, mites or deer flies, by handling or ingestion of infected material and/or water, and by inhalation of contaminated dust particles. The microbes are small, non-motile, non-sporulating gram-negative coccobacilli, which are nutritionally fastidious and require cysteine or Na-thioglycolate as reducing agent for their growth in vitro [28,29]. According to its lifestyle, *F. tularensis* is a facultative intracellular bacterium proliferating in monocyte-macrophage cells and hepatocytes.

Originally, two main types (genotypes, biovars) of *F. tularensis* were distinguished: *F. tularensis tularensis* occurring in the New World, and *F. tularensis palaearctica* (*holarctica*) spread over whole northern hemisphere, tentatively also designated as type ''A'' and ''B'', respectively [30,31]. In addition two other biovars (subspecies), *F. tularensis palaearctica* subvar *japonica* and *F. tularensis* biovar *mediaasiatica* were proposed later on [32]. The intraspecies position of last two sub-variants of *F. tularensis* remains to be definitely specified. *F. tularensis mediaasiatica* seems to be included in subsp. *tularensis* (type A) and subvar *japonica* form rather separate subspecies [33,34].

In 1940s, Gajskij repeatedly cultured the *F. tularensis* biovar *holarctica* on artificial media supplemented with antiserum [35]. Of several attenuated strains, such as Moscow, Ondatra IV, 155, and 10, Elbert consequently used strain No. 15 for construction of live vaccine against tularemia (for review, see Ref. [36]). After re-isolation of individual colonies derived from vaccine produced in the Gamaleia Institute, Eigelsbach and Downs gave rise the live vaccine strain designated LVS in 1950s [37] (for review, see Ref. [38]). This LVS strain was used for vaccination in the USSR and Eastern Europe, and for protection of laboratory workers at USAMRIID, Fort Detrick, Frederick, MD, USA. The data presented in research reports from the USSR and Czechoslovak laboratories and retrospective study of laboratory-acquired tularemia done in the 1970s documented the partial protective effect of live vaccines against virulent tularemic strains [39–41]. Both strains Gajskij 15 and LVS are infectious for mice eliciting disease, which is in its main characteristics similar to human tularemia. The experimental murine tularemia induced by attenuated strains has been broadly used as the model for the study of pathogenesis of tularemia and studies of innate and adaptive immune mechanisms which are prerequisite for the expression of the protective effect of vaccination and natural infection against subsequent challenge [42,43].

Human tularemia is a sub-acute, usually moderately severe anthropo-zoonotic disease. The clinical manifestation of the infection seems to be dependent on the route of transmission, the genetic background of the host, and the virulence of the infecting microorganism. Ulceroglandular or glandular, oculoglandular, oropharyngeal, typhoidal, and respiratory (pneumonic) tularemia are distinct forms of tularemia according to the portal of entry. In general, infected patients expressing arbitrary form of tularemia may have rapid onset of fever accompanied with cough, creation of granulomas and in the case of more severe disease, with secondary pneumonia, which might be attributed to a transient bacteriemia. Mortality rates range from less than 1% in Eurasia, where the subspecies *F. tularensis holarctica* with inherent lower virulence are endemic, to 30% in North America, where the subspecies *F. tularensis tularensis* causes the most severe pulmonary disease.

F. tularensis should accomplish several essential events to overcome the structural barriers lying between the microbe and the intracellular niche suitable for its proliferation. Microbes must first adher to the target cell followed by entering the host cells by the process called induced phagocytosis. The precise molecular base of this process is currently

unknown. Once internalized, *F. tularensis* microbes proliferate inside the cells. Likewise the process of cell entering, the compartment, where the microbes undergo intracellular multiplication has been poorly defined until now. Nevertheless, at least in the early stages of bacteria–macrophage interaction, the compartment can be characterized as an acidic vesicle that facilitates high input of iron essential for the growth of *F. tularensis* microbes [44]. In in vitro macrophage model system, intracellular multiplication of *F. tularensis* resulted in cytopathogenic effect [45] accompanied by microbe-induced apoptosis of infected cultured cells [46]. The induced programmed destruction of infected cell can ensure the transit of bacterium from a disrupted ''exhausted'' cell to a different one, originally an uninfected cell. The mechanism by which the tularemia microbe induces the fatal end of an infected host cell remains obscure because, unlike many other intracellular bacteria, *F. tularensis* has no toxic LPS and does not produce any known toxin.

Macrophages, which are targeted by *F. tularensis* microbes, were identified to also be key cells in the process of resolution of infection. The elimination of bacteria from tissues of an infected host is highly dependent on the mechanisms of innate as well as adaptive immunity, both under multigenic control. The immunoregulatory pathway of Th1 type characterized by IFN-γ, TNF-α and IL-2 production is preferentially activated in the course of primary infections of experimental animal models and after vaccination of human beings with live vaccine strain [47,48].

2. Proteome analysis of *F. tularensis* LVS

2.1. Construction of reference protein maps of whole cell lysates

As mentioned above, the formation of comprehensive 2-DE microbial databases is prerequisite for subsequent comparative studies of vaccine and virulent strain protein profiles, for the analysis of the phenotype of multigenic responses invoked, for example, by cultivation of microbes under stressful conditions and, finally, for confirmation of the translation of the predicted ORFs. The construction of 2-DE *F. tularensis* reference maps is based mostly on the analysis of total cellular proteins extracted from vaccine strain *F. tularensis* LVS. The solubilized proteins were separated on 2-DE gels of two different immobilized pH gradients (IPG). The second dimension separation on gradient 9–16% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or 16.5% T, 6% C tricine SDS–PAGE then allowed resolution of proteins with molecular weights (MW) ranging from 8 to 200 kDa and 5 to 49 kDa, respectively. To increase the number of proteins entering the gels, the cell extracts were dissolved in isoelectric focusing (IEF) buffer containing thiourea and tributylphosphine which improve protein solubilization for 2-DE [49], and 150 μg of protein was loaded overnight by in-gel rehydration [50]. Using a broad range pH 3–10 IPG strips, more than 1500 spots were detected (Fig. 1). Application of the wide pH range gradient usually leads to optimal resolution of proteins with p*I* values from 4.0 to 7.5; however, this gradient fails to perform adequate separation of more basic spots. Therefore, an additional overlapping gradient of pH 6–11 was utilized for construction of 2-DE reference map of basic tularemic proteins. As can be seen from Fig. 2, the application of basic pH gradient did not significantly increase the number of detected basic spots in comparison to the wide range pH gradient, nevertheless the basic proteins were much better resolved because they were allowed to reach positions corresponding to their p*I* values. Using basic range IPG strip of pH 6–11, the separation of ~500 spots was repeatedly accomplished. The application of tricine SDS–PAGE [51] in the second dimension was aimed improving the resolution of low molecular weight proteins normally hidden in the bottom part of the gel containing the mixture of ampholytes and detergents. The reference tricine SDS–PAGE gel containing ~1000 spots is shown in Fig. 3. Compared to classical gradient 9–16% SDS–PAGE (Fig. 1), tricine SDS–PAGE gel provides very good resolution of proteins with molecular weights from 3 to 30 kDa.

Recently, we got access to complete cell lysates of *F. tularensis* type A highly virulent strains. Availability of this material enabled comparative proteomic studies of type A and type B strains (see below) to be started and a reference 2-DE map of this most

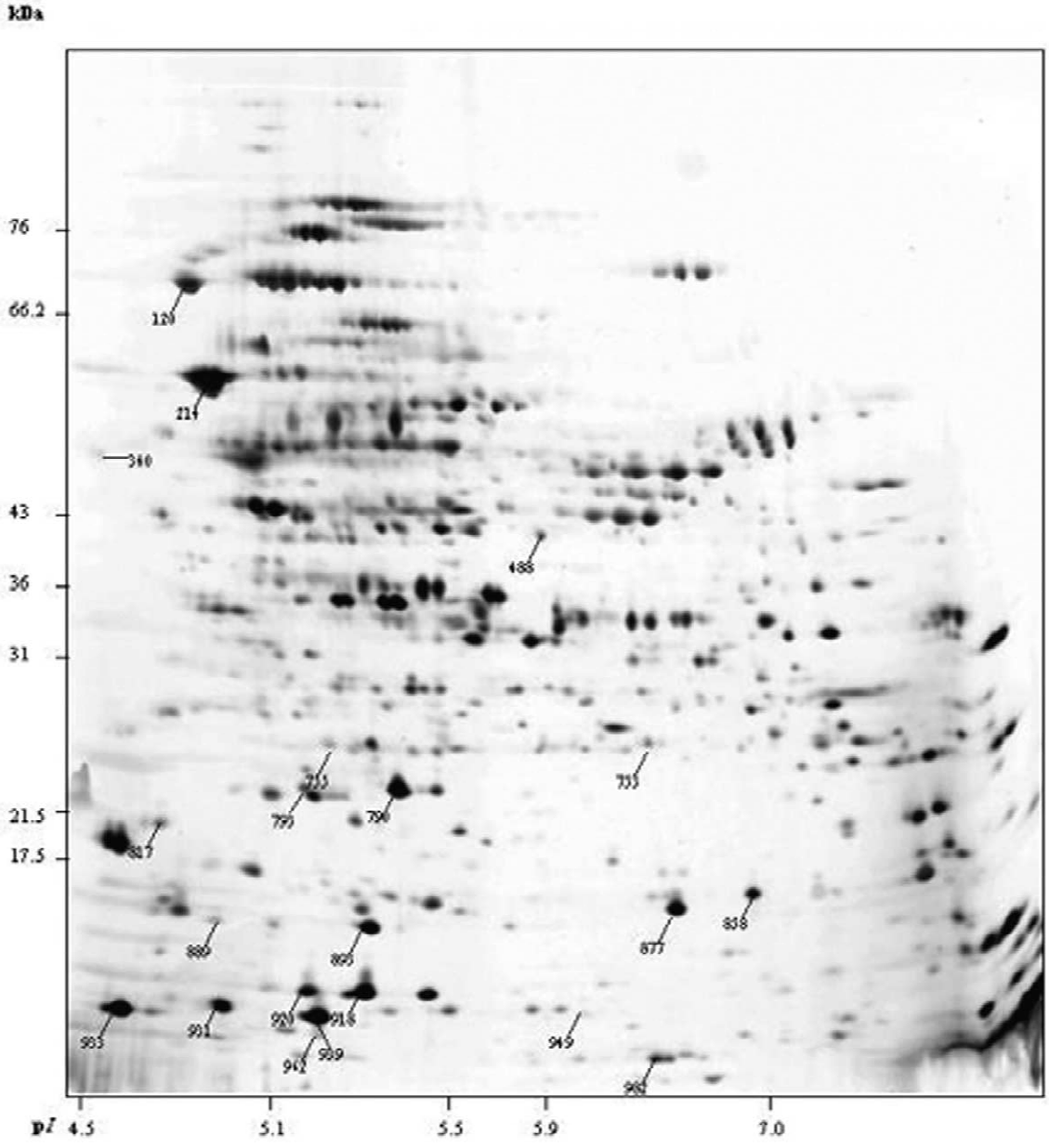

Fig. 1. Silver-stained 2-DE reference map of *F. tularensis* LVS. Extracted proteins were resolved by IEF in the pH range 3–10 followed by SDS–PAGE gradient gel (9–16%). The spot numbers indicate identified proteins.

virulent strain to be constructed. The reference map of *F. tularensis* type A strain is shown in Fig. 4. This wild type strain, designated 176, was isolated from a blood sample of a patient with tularemia and belongs to subspecies *F. tularensis tularensis*.

The proteins indicated in all 2-DE reference maps by their spot numbers were identified by peptide mass fingerprinting. When the genome sequence of *F. tularensis* was not accessible the obtained mass spectra were matched against SWISS-PROT or

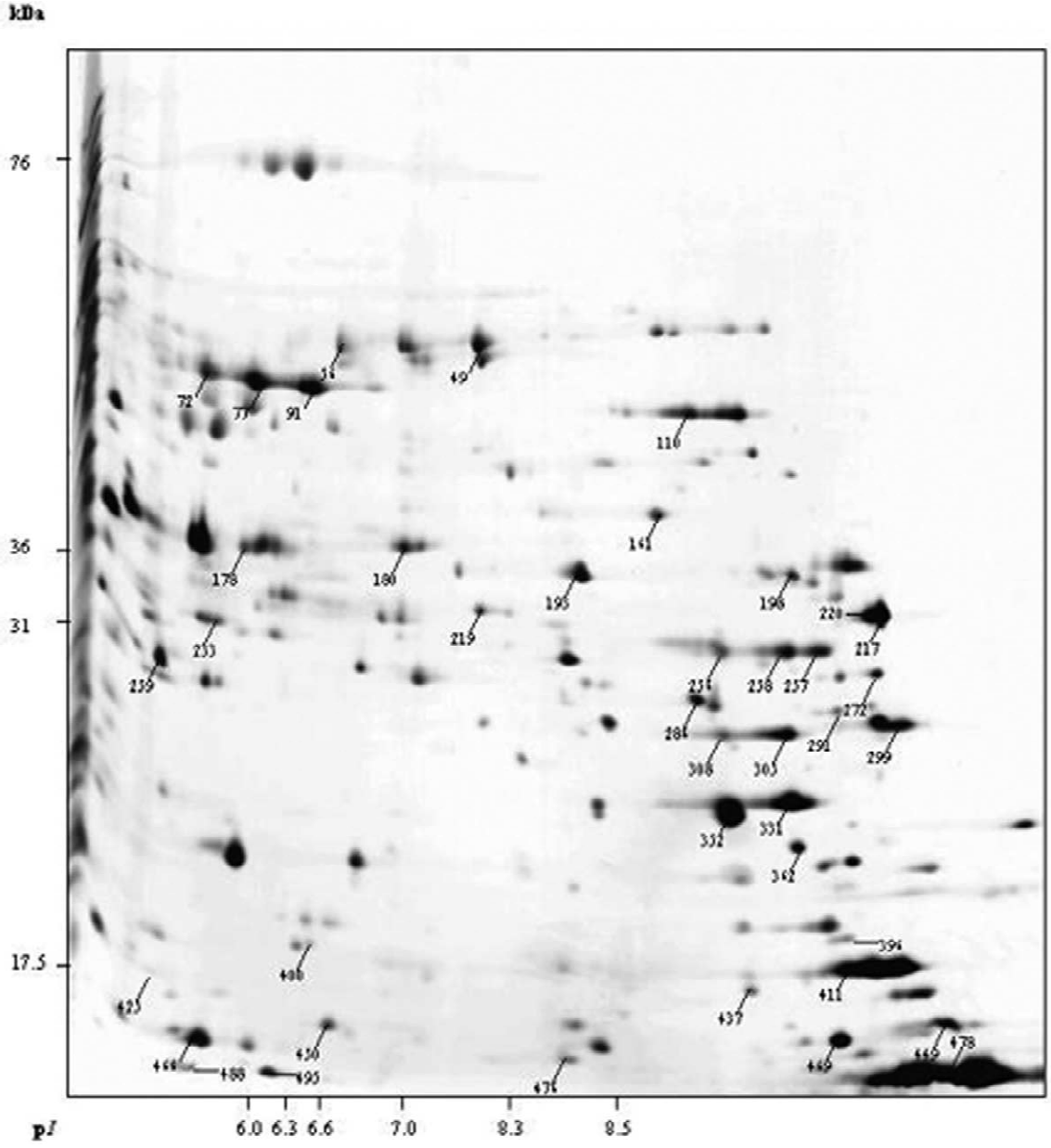

Fig. 2. Silver-stained 2-DE reference map of *F. tularensis* LVS. Extracted proteins were resolved by IEF in the pH range 6–11 followed by SDS–PAGE gradient gel (9–16%). The spot numbers indicate identified proteins.

NCBI protein databases where amino acid sequences of ~80 tularemic proteins are located. This approach was not very successful, resulting in unambiguous identification of only 19 different protein species. All data acquired from this search are summarized in Table 1. Most identified proteins belong to the molecular chaperone system of *F. tularensis*. It is interesting to note that several charge and mass variants of the 10-kDa chaperonin were identified on 2-DE maps of both vaccine and highly virulent strain

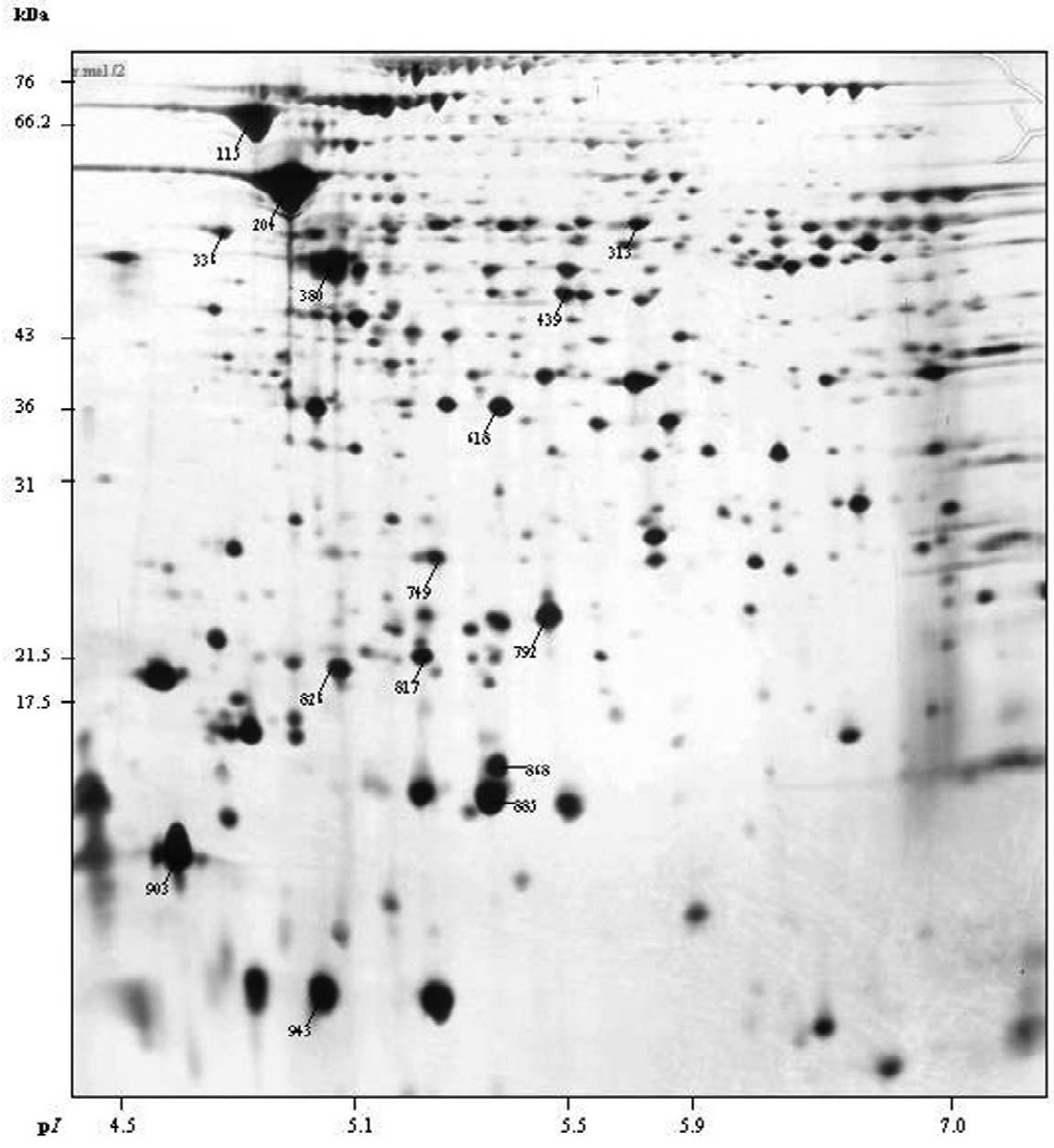

Fig. 3. Silver-stained 2-DE reference map of *F. tularensis* LVS. Extracted proteins were resolved by IEF in the pH range 3–10 followed by tricine SDS–PAGE gel (16.5% T, 6% C). The spot numbers indicate identified proteins.

176. While the MW/p*I* 9.7/4.9 10-kDa chaperonin variant was common for both types of strains, the distribution of the others in vaccine and virulent strain can be clearly distinguished. At the end of 2001 the annotation of partial genome sequence of *F. tularensis* strain Schu 4 was published [52]. In total 1804 candidate ORFs were identified in the data set of which 1289 were thought to encode proteins (743 genes with known function, 133 genes coding for hypothetical proteins and 413 genes with no database match).

Currently, the protein database created by transla-

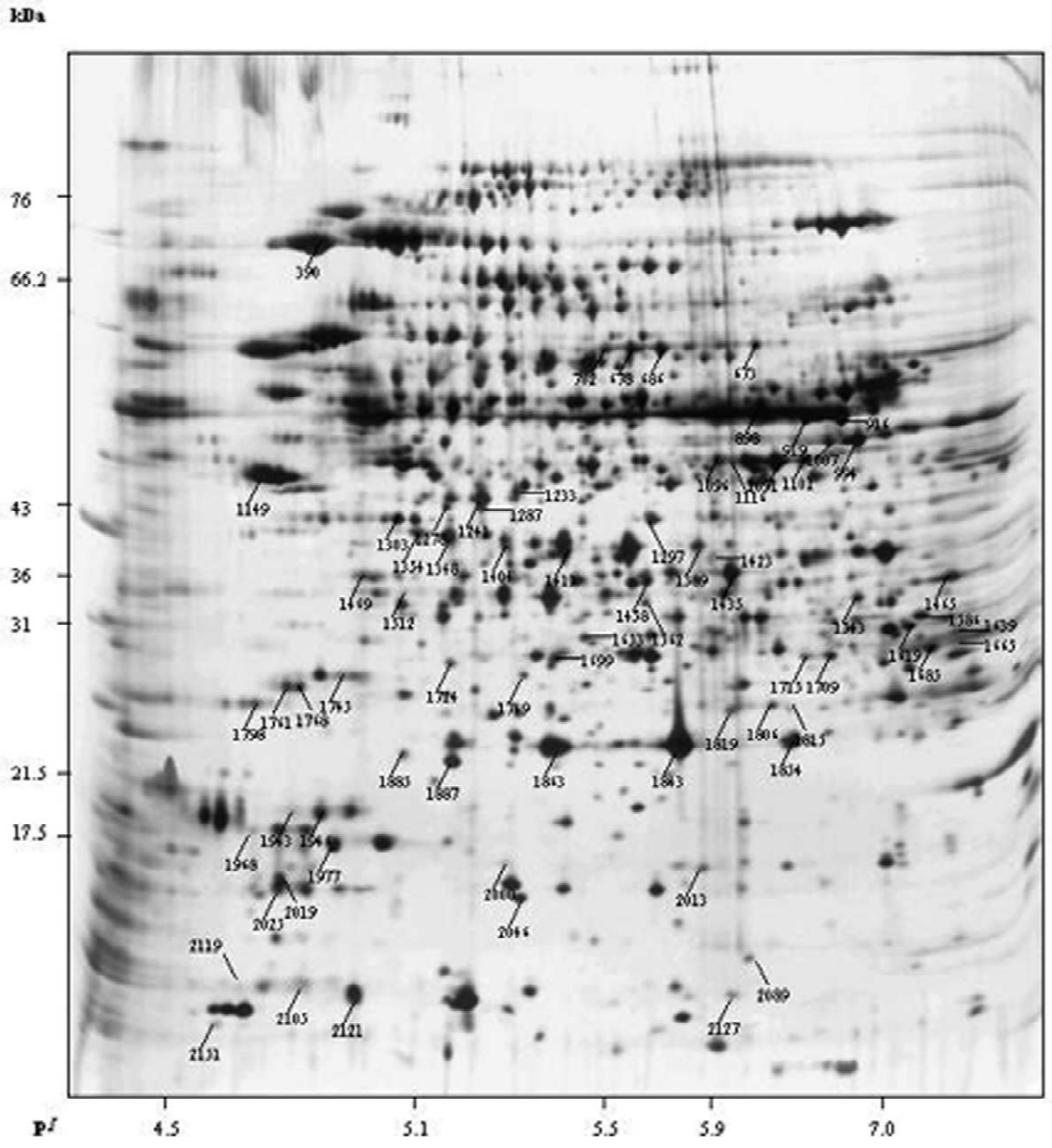

Fig. 4. Silver-stained 2-DE reference map of wild type *F. tularensis* strain, designated 176. Extracted proteins were resolved by IEF in the pH range 3–10 followed by SDS–PAGE gradient gel (9–16%). The spot numbers indicate identified proteins.

tion of all possible ORFs is available for matching process. The amino acid sequences obtained from the ORF database are utilized in the search for proteins with sequence homology of other microbial species listed in the NCBI database using the BLAST program. By this approach 124 proteins have so far been newly annotated. The basic characteristics of identified proteins together with the source of their homologues are listed in Table 2. Among identified spots only one protein (spot no. 943, tricine SDS–

Table 1
The list of identified proteins of *F. tularensis* included in the NCBI and SWISSPROT databases

Spot No.	Protein name	Accession No.	MW (kDa)/p*I*, theoretical	MW (kDa)/p*I*, measured	Sequence coverage, %
		Whole-cell antigen *F. tularensis* LVS IEF pH 3–10 9–16% SDS–PAGE			
120	Chaperone protein dnaK	P48205	69.2/4.9	69.2/4.9	13
214	60-kDa Chaperonin	P94798	57.4/5.0	55.6/4.9	30
790	Hypothetical 23-kDa protein	CAA70085	22.1/5.7	23.3/5.4	40
793	Hypothetical 23-kDa protein	CAA70085	22.1/5.7	23.3/5.2	24
920	10-kDa Chaperonin	P94797	10.3/5.5	10.3/5.2	32
931	10-kDa Chaperonin	P94797	10.3/5.5	9.7/4.9	20
939	10-kDa Chaperonin	P94797	10.3/5.5	9.3/5.3	38
962	10-kDa Chaperonin	P94797	10.3/5.5	8.6/5.3	20
		Whole-cell antigen *F. tularensis* LVS IEF pH 6–11 9–16% SDS–PAGE			
488	Putative peptidyl-prolyl *cis-trans* isomerase	AAG33118	10.2/6.5	14.6/5.3	64
495	Putative peptidyl-prolyl *cis-trans* isomerase	AAG33118	10.2/6.5	14.5/6.1	58
		Whole-cell antigen *F. tularensis* LVS IEF pH 3–10 16.5% T, 6% C tricine SDS–PAGE			
115	Chaperone protein dnaK	P48205	69.2/4.9	69.2/4.9	26
204	60-kDa Chaperonin	P94798	57.4/5.0	60.3/4.9	37
792	Hypothetical 23-kDa protein	CAA70085	22.1/5.7	23.0/5.5	18
903	Macrophage growth locusB	AAC29033	15.1/4.1	7.8/4.7	16
		Integral membrane proteins *F. tularensis* LVS IEF pH 3–10 9–16% SDS–PAGE			
47	60-kDa Chaperonin	P94798	57.4/5.0	57.4/5.0	14
236	Hypothetical 23-kDa protein	CAA70085	22.1/5.7	23.0/5.4	34
275	17-kDa Major membrane protein (precursor)	P18149	16.0/9.2	13.3/4.8	56
		Whole-cell antigen *F. tularensis* subsp. *tularensis*, strain 176 IEF pH 3–10 9–16% SDS–PAGE			
390	Chaperone protein dnaK	P48205	69.2/4.9	69.2/4.8	28
673	Acid phosphatase	AAB06624	57.6/5.9	59.0/6.0	23
1149	Cell division protein FtsZ	AAC99558	39.7/4.8	46.9/4.7	26
1745	Triosephosphate isomerase	P96763	27.6/4.9	27.4/4.9	24
1761	GrpE protein	P48204	22.0/4.8	26.6/4.8	40
1768	GrpE protein	P48204	22.0/4.8	26.6/4.8	26
1843	Hypothetical 23-kDa protein	CAA70085	22.1/5.7	23.0/5.8	45
1854	Hypothetical 23-kDa protein	CAA70085	22.1/5.7	23.0/6.0	30
1863	Hypothetical 23-kDa protein	CAA70085	22.1/5.7	23.0/5.5	24
2105	10-kDa Chaperonin	P94797	10.3/5.5	10.0/4.8	20
2121	10-kDa Chaperonin	P94797	10.3/5.5	9.7/4.9	21
2127	10-kDa Chaperonin	P94797	10.3/5.5	9.7/5.9	37

The mass spectra were recorded in reflector mode on a MALDI mass spectrometer Voyager-DE STR (Perseptive Biosystems, Framingham, MA, USA) equipped with delayed extraction. Proteins were identified by peptide mass fingerprinting using ProFound and PeptIdent programs. The spectra of integral membrane proteins were recorded on a LC–MS–MS mass spectrometer Q-TOF Ultima™ API (Micromass, UK) fitted with a Z-spray atmospheric pressure ionization (API). The in-gel protein digest samples were separated by means of a Micromass modular CapLC system (Micromass, UK) connected directly to the Z-spray source. The MS–MS spectra were acquired on the four most intense ions. All data were processed automatically by means of ProteinLynx software. Protein identification was done by searching the Non Redundant Data Base (NRDB) using the ProteinLynx Global Server engine.

Table 2
The list of identified proteins of *F. tularensis*-homologues according to BLAST

Spot No.	MW (kDa)/p*I*, theoretical	MW (kDa)/p*I*, measured	Sequence coverage, %	Protein homologues according to BLAST		*E* value
				Protein name Organism	Accession No.	
			Whole-cell antigen *F. tularensis* LVS IEF pH 3–10 9–16% SDS–PAGE			
340	41.8/5.0	47.8/4.6	30	Elongation factor TU *S. typhimurium*	P21694	1×10^{-168}
488	39.6/5.7	40.3/5.9	33	Glycine-cleavage system protein T1 *Pseudomonas aeruginosa*	G82994	2×10^{-97}
753	26.0/6.1	25.7/6.3	22	Oxidoreductase *Vibrio cholerae*	G82383	2×10^{-84}
755	25.7/7.7	25.6/5.3	23	Hypothetical protein *Plasmodium falciparum*	CAB38995	0.12
817	23.3/5.2	20.3/4.7	23	Peptidoglycan-associated lipoprotein Pal *Yersinia pestis*	CAC89968	3×10^{-20}
858	18.2/6.8	15.3/6.8	32	(3*R*)-hydroxymyristoyl (acyl carrier protein) dehydratase *P. aeruginosa*	AAG07033	3×10^{-31}
877	18.8/7.7	14.4/6.4	34	Cationic 19-kDa outer membrane protein precursor *Yersinia enterocolitica*	P31519	2×10^{-11}
889	16.5/5.0	13.7/4.9	42	Biotin carboxyl carrier protein *P. aeruginosa*	P37799	6×10^{-27}
893	16.1/5.6	13.3/5.4	45	50S Ribosomal protein L9 *P. aeruginosa*	F83029	2×10^{-30}
918	18.5/5.3	10.3/5.4	40	Probable bacterioferritin *P. aeruginosa*	B83036	3×10^{-4}
933	12.8/4.6	9.6/4.6	20	50S Ribosomal protein *Campylobacter jejuni*	H81392	3×10^{-25}
949	16.3/6.1	9.4/6.0	42	3-Dehydroquinase *Buchnera* sp.	P57479	5×10^{-30}
982	11.2/5.9	7.8/6.3	46	Probable sigma (54) modulation protein *Pseudomonas putida*	P15592	2×10^{-23}
			Whole-cell antigen *F. tularensis* LVS IEF pH 6–11 9–16% SDS–PAGE			
49	52.1/7.2	52.9/7.9	33	Inosine-5′-monophosphate dehydrogenase *P. aeruginosa*	H83173	1×10^{-172}
56	52.1/7.2	52.5/6.7	27	Inosine-5′-monophosphate dehydrogenase *P. aeruginosa*	H83173	1×10^{-172}
72	49.2/6.5	50.1/5.4	32	Glutamate dehydrogenase *Clostridium symbiosum*	P24295	1×10^{-164}
77	49.2/6.5	49.2/6.0	32	Glutamate dehydrogenase *C. symbiosum*	P24295	1×10^{-164}
91	49.2/6.5	48.5/6.6	32	Glutamate dehydrogenase *C. symbiosum*	P24295	1×10^{-164}
110	48.1/8.6	46.2/8.6	12	Penicillin-binding protein 5 *P. aeruginosa*	C83146	1×10^{-65}

Table 2. Continued

Spot No.	MW (kDa)/p*I*, theoretical	MW (kDa)/p*I*, measured	Sequence coverage, %	Protein homologues according to BLAST		*E* value
				Protein name Organism	Accession No.	
161	39.9/8.5	37.9/8.6	16	Hypothetical protein jhp1045 *H. pylori*	G71856	5×10^{-5}
178	36.1/6.4	36.0/5.8	27	Malate dehydrogenase *Sinorhizobium melioti*	AAG41996	1×10^{-108}
180	36.1/6.4	36.0/7	31	Malate dehydrogenase *S. melioti*	AAG41996	1×10^{-108}
193	35.5/7.7	34.4/8.4	22	Acetyl-coenzyme A carboxylase *Pasteurella multocida*	AAK02376	1×10^{-101}
198	33.7/9.1	34.3/8.8	15	Curved DNA-binding protein cbpA *E. coli*	P36659	4×10^{-34}
217	24.6/9.5	32.1/9.0	17	50S Ribosomal subunit protein L1 *E. coli*	P02384	4×10^{-77}
219	32.0/7.7	32.2/7.9	35	IMI-1 *Enterobacter cloacae*	AAA93461	2×10^{-45}
220	24.6/9.5	31.9/8.9	24	50S Ribosomal subunit protein L1 *E. coli*	P02384	4×10^{-77}
233	27.9/6.2	31.7/5.6	40	Probable thiosulfate sulfurtransferase *P. aeruginosa*	F83319	4×10^{-11}
256	26.6/7.9	30.0/8.7	22	Succinate dehydrogenase putative iron sulphur subunit *Shewanella frigidimarina*	CAA74088	4×10^{-95}
257	27.7/8.9	30.0/8.9	52	DNA mismatch repair protein msh2 *Schizosaccharomyces pombe*	T43699	0.017
258	32.0/9.0	30.0/8.8	11	LPSA protein *Dichelobacter nodosus*	AAC43386	7×10^{-62}
259	25.5/6	29.7/5.0	38	Probable two-component response regulator *P. aeruginosa*	F83048	8×10^{-43}
272	30.3/6.6	28.9/9.0	20	L-Aspartate beta-decarboxylase *Alcaligenes faecalis* subsp. *faecalis*	AAK58507	6×10^{-73}
286	27.7/8.9	27.6/8.6	50	DNA mismatch repair protein msh2 *S. pombe*	T43699	0.017
291	23.4/9.3	27.1/8.9	23	Dephospho-coenzyme A kinase *H. influenzae*	P44920	2×10^{-22}
299	23.1/9.5	26.4/9.0	38	50S Ribosomal protein L3 *H. influenzae* (strain Rd KW20)	E64092	5×10^{-74}
305	24.4/9.0	26.0/8.8	22	Pyrrolidone-carboxylate peptidase *Staphylococcus aureus* subsp. *aureus* Mu50	BAB43788	1×10^{-46}
308	24.4/9.0	25.9/8.7	17	Pyrrolidone-carboxylate peptidase *S. aureus* subsp. *aureus* Mu50	BAB43788	1×10^{-46}
331	21.0/8.5	23.1/8.8	21	Conserved hypothetical protein *Y. pestis*	AJ414148	4×10^{-18}
352	21.0/8.5	22.4/8.7	35	Conserved hypothetical protein *Y. pestis*	AJ414148	4×10^{-18}
362	20.0/8.7	21.4/8.8	15	Transcription antitermination protein NusG *P. aeruginosa*	AAG07663	2×10^{-51}
396	19.6/9.4	18.3/8.9	28	Peptide methionine sulfoxide reductase *V. cholerae*	C82439	3×10^{-54}
400	15.8/6.2	18.1/6.5	21	ATP synthase *Bacilus subtilis*	P37812	1×10^{-14}
411	14.4/9.4	17.3/8.9	41	RpS8 *P. multocida*	AAK03485	6×10^{-37}

Table 2. Continued

Spot No.	MW (kDa)/p*I*, theoretical	MW (kDa)/p*I*, measured	Sequence coverage, %	Protein homologues according to BLAST		*E* value
				Protein name Organism	Accession No.	
425	16.3/6.1	17.2/4.9	50	3-Dehydroquinase *Buchnera* sp.	P57479	5×10^{-30}
437	14.0/9.3	16.7/8.7	18	Conserved hypothetical protein VC1812 *V. cholerae*	H82154	5×10^{-6}
449	11.9/9.8	15.7/9.1	31	Ribosomal protein S10 *V. cholerae*	G82059	3×10^{-45}
450	16.1/6.6	15.7/6.6	36	Hypothetical protein T0H1.4 *Caenorhabditis elegans*	T24480	0.019
466	11.2/5.9	15.4/5.4	60	Hypothetical protein 102 *P. putida*	T01754	2×10^{-23}
469	10.8/9.5	15.3/8.9	19	50S Ribosomal protein L25 *Xylella fastidiosa*	D82532	5×10^{-22}
476	12.2/7.7	14.8/8.4	37	Thioredoxin *Cyanidium caldarium*	P37395	1×10^{-22}
478	9.5/9.8	14.5/9.1	35	Histone-like protein HU form B *P. putida*	AAK52475	3×10^{-27}
			Whole-cell antigen *F. tularensis* LVS IEF pH 3–10 16.5% T, 6% C tricine SDS–PAGE			
313	50.5/5.6	55.4/5.8	12	Pyruvate dehydrogenase *V. cholerae*	B82079	1×10^{-170}
336	49.5/4.7	54.3/4.8	14	Enolase *Borrelia burgdorferi*	O51312	1×10^{-153}
380	41.8/5.0	50.4/5.0	19	Elongation factor Tu *S. typhimurium*	P21694	1×10^{-168}
439	38.1/5.3	47.3/5.5	14	Fructose-bisphosphate aldolase *Pseudomonas stutzeri*	O87796	1×10^{-169}
618	31.0/5.6	36.7/5.4	22	Elongation factor TS *P. multocida*	P57983	6×10^{-77}
749	25.7/7.7	26.2/5.3	38	Hypothetical protein *P. falciparum*	CAB38995	0.12
817	21.9/5.4	21.9/5.3	29	Superoxide dismutase *E. coli*	P09157	1×10^{-73}
826	19.7/5.1	20.5/5.1	18	Conserved hypothetical protein *Sinorhizobium meliloti*	CAC47046	1×10^{-65}
868	16.1/5.6	12.2/5.4	13	50S Ribosomal protein L9 *P. aeruginosa*	F83029	3×10^{-30}
885	18.5/5.3	10.3/5.4	44	Probable bacterioferritin *P. aeruginosa*	B83036	3×10^{-4}
943	8.6/4.5	3.6/5.0	59	Unknown protein	–	–
			Whole-cell antigen *F. tularensis* subsp. *tularensis*, strain 176 IEF pH 3–10 9–16% SDS–PAGE			
678	57.6/5.8	58.7/5.7	19	Phosphoglyceromutase *S. typhimurium* LT2	NP_462604	1×10^{-171}

Table 2. Continued

Spot No.	MW (kDa)/p*I*, theoretical	MW (kDa)/p*I*, measured	Sequence coverage, %	Protein homologues according to BLAST		*E* value
				Protein name Organism	Accession No.	
686	57.6/5.8	58.7/5.8	19	Phosphoglyceromutase *S. typhimurium* LT2	NP_462604	1×10^{-171}
702	57.6/5.8	58.6/5.6	19	Phosphoglyceromutase *S. typhimurium* LT2	NP_462604	1×10^{-171}
898	48.6/6.1	52.8/6.0	16	Serine tRNA synthetase *E. coli*	AAG55380	1×10^{-143}
916	49.1/6.5	52.4/6.4	12	NAD-specific glutamate dehydrogenase *C. symbiosum*	P24295	1×10^{-161}
919	49.1/6.5	52.4/6.1	22	NAD-specific glutamate dehydrogenase *C. symbiosum*	P24295	1×10^{-164}
994	50.3/6.4	50.2/6.5	13	orf, hypothetical protein *E. coli* O157:H7 EDL933	NP_288589	1×10^{-153}
1007	50.5/5.6	50.2/6.3	11	Pyruvate dehydrogenase, E3 component, lipoamide dehydrogenase *V. cholerae*	NP_232042	1×10^{-170}
1091	39.7/6.0	48.4/6.0	11	Probable ABC transporter membrane protein (ycf24) *Nostoc* sp. PCC 7120	NP_486532	1×10^{-159}
1096	47.0/6.2	48.5/5.9	13	Citrate synthase *P. aeruginosa*	NP_250271	1×10^{-120}
1101	47.0/6.2	48.5/6.1	18	Citrate synthase *P. aeruginosa*	NP_250271	1×10^{-120}
1116	47.0/6.2	48.4/5.9	13	Citrate synthase *P. aeruginosa*	NP_250271	1×10^{-120}
1233	38.2/5.3	44.7/5.4	24	Fructose-bisphosphate aldolase *P. stutzeri*	O87796	1×10^{-169}
1241	38.2/5.3	43.9/5.3	20	Fructose-bisphosphate aldolase *P. stutzeri*	O87796	1×10^{-169}
1278	41.9/5.5	42.9/5.2	13	Phosphoglycerate kinase *E. coli* K12	NP_417401	1×10^{-127}
1287	38.2/5.3	42.6/5.3	20	Fructose-bisphosphate aldolase *P. stutzeri*	O87796	1×10^{-169}
1297	41.0/5.7	41.6/5.7	12	Hypothetical transmembrane protein *Ralstonia solanacearum*	NP_521198	3×10^{-39}
1303	39.0/5.4	41.5/5.2	30	Glycerophosphoryl diester phosphodiesterase *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586	NP_602691	3×10^{-18}
1348	38.3/5.3	39.7/5.2	22	Glutamine synthetase, chloroplast precursor *Chlamydomonas reinhardtii* (eukaryota)	Q42689	4×10^{-97}
1354	38.3/5.3	40.0/5.1	30	Glutamine synthetase, chloroplast precursor *C. reinhardtii* (eukaryota)	Q42689	4×10^{-97}
1389	37.8/5.9	38.6/5.9	13	UDP-glucose 4-epimerase *B. subtilis*	NP_391765	1×10^{-116}
1404	37.6/6.3	38.4/5.4	39	Glyceraldehyde-3-phosphate dehydrogenase *Clostridium perfringens*	NP_562220	1×10^{-129}
1413	38.3/5.3	37.8/5.5	11	S-adenosylmethionine:tRNA ribosyltransferase-isomerase *V. cholerae*	NP_230388	2×10^{-90}
1423	36.1/6.4	37.6/5.9	22	Malate dehydrogenase *Agrobacterium tumefaciens*	NP_533304	1×10^{-109}
1435	30.1/6.1	36.1/5.9	21	Putative succinyl-CoA synthetase alpha subunit *Neisseria meningitidis* Z2491	NP_283922	1×10^{-108}

Table 2. Continued

Spot No.	MW (kDa)/p*I*, theoretical	MW (kDa)/p*I*, measured	Sequence coverage, %	Protein homologues according to BLAST		*E* value
				Protein name Organism	Accession No.	
1458	35.5/5.8	35.5/5.7	17	Chain A, the crystal structure of the thermophilic carboxylesterase Est2 *Alicyclobacillus acidocaldarius*	1EVQ_A	3×10^{-31}
1465	35.5/7.7	35.6/8.9	30	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha *Y. pestis*	NP_404673	1×10^{-102}
1469	33.5/5.1	35.7/5.1	22	FabD *P. multocida*	NP_246854	2×10^{-75}
1512	32.8/5.2	34.3/5.2	16	DdlB *P. multocida*	NP_245081	1×10^{-61}
1543	32.2/6.6	33.7/6.5	12	UTP-glucose-1-phosphate uridylyltransferase *N. meningitidis* MC58	NP_273681	8×10^{-82}
1562	32.4/5.7	33.3/5.7	17	Probable hydratase *P. aeruginosa*	NP_248984	6×10^{-89}
1586	32.0/7.7	32.2/8.1	25	IMI-1 *E. cloacae*	AAA93461	3×10^{-45}
1619	30.1/6.9	31.3/7.7	18	Septum site-determining protein *Y. pestis*	NP_405629	2×10^{-77}
1639	30.1/6.9	30.9/9.1	26	Septum site-determining protein *Y. pestis*	NP_405629	2×10^{-77}
1653	27.8/5.8	30.3/5.5	19	Hypothetical protein Rv3404c *M. tuberculosis* H37Rv	NP_217921	4×10^{-42}
1665	31.3/7.7	30.1/9.1	18	Probable thymidilate synthase *R. solanacearum*	NP_519068	1×10^{-102}
1685	30.3/6.6	29.5/8.4	12	L-aspartate beta-decarboxylase *A. faecalis* subsp. *faecalis*	AAK58507	7×10^{-73}
1699	27.9/6.2	28.9/5.5	20	AGR_pTi_bx104p *A. tumefaciens* str. C58 (*Cereon*)	NP_396608	3×10^{-11}
1709	28.4/6.4	28.9/6.3	28	Methionine aminopeptidase *P. aeruginosa*	NP_252347	1×10^{-102}
1715	28.4/6.4	28.8/6.1	28	Methionine aminopeptidase *P. aeruginosa*	NP_252347	1×10^{-102}
1724	29.1/5.4	28.3/5.2	19	Tryptophan synthase alpha chain *Vibrio parahaemolyticus*	P22095	1×10^{-88}
1749	25.2/5.6	27.4/5.4	31	Hypothetical protein *Synechocystis* sp. PCC 6803	NP_440208	2×10^{-54}
1798	23.7/4.6	25.4/4.7	20	Sigma cross-reacting protein 27A *V. cholerae*	NP_232999	2×10^{-46}
1806	26.0/6.1	25.3/6.0	29	Oxidoreductase, short-chain dehydrogenase/reductase family *V. cholerae*	NP_233439	2×10^{-84}
1815	23.3/6.1	25.1/6.0	28	Thymidylate kinase *V. cholerae*	NP_231650	2×10^{-44}
1819	23.4/6.0	25.0/5.9	33	Orotidine 5′-phosphate decarboxylase *Buchnera* sp. APS	NP_240094	2×10^{-17}
1885	21.9/5.4	22.4/5.2	47	Superoxide dismutase *Salmonella enterica* subsp. *enterica* serovar *typhi*	NP_456099	1×10^{-73}
1887	21.9/5.4	21.9/5.3	47	Superoxide dismutase *S. enterica* subsp. *enterica* serovar *typhi*	NP_456099	1×10^{-73}

Table 2. Continued

Spot No.	MW (kDa)/p*I*, theoretical	MW (kDa)/p*I*, measured	Sequence coverage, %	Protein homologues according to BLAST		*E* value
				Protein name Organism	Accession No.	
1943	19.6/4.9	18.8/4.8	17	Chain A, structure of inorganic pyrophosphatase *E. coli*	1JFD_A	3×10^{-60}
1946	19.6/4.9	18.7/4.9	28	Inorganic pyrophosphatase *Y. pestis*	CAC92750	3×10^{-60}
1968	23.3/5.2	17.8/4.7	20	Peptidoglycan-associated lipoprotein Pal *Y. pestis*	CAC89968	4×10^{-20}
1977	19.7/5.1	17.0/4.9	31	Conserved hypothetical protein 10 *Rhodobacter capsulatus*	S39907	3×10^{-64}
2000	16.3/6.6	15.7/5.3	18	Alcohol dehydrogenase-like protein *Arabidopsis thaliana*	BAB01821	6×10^{-9}
2013	15.5/5.9	15.2/5.9	31	Nucleoside diphosphate kinase *X. fastidiosa* 9a5c	NP_297748	2×10^{-54}
2019	17.7/4.8	14.8/4.8	41	Transcription elongation factor *S. enterica* subsp. *enterica* serovar *typhi*	NP_457678	8×10^{-46}
2025	14.0/5.6	14.0/4.8	25	Hypothetical protein MAL4P2.01 *P. falciparum* 3D7	CAB62842	9×10^{-4}
2046	16.1/5.6	13.3/5.4	45	50S Ribosomal protein L9 *P. aeruginosa*	F83029	3×10^{-30}
2089	11.0/6.7	10.8/6.0	23	Transport protein homolog *Arthrobacter* sp. strain TE1826	T44249	1.2
2119	10.9/5.7	9.9/4.7	13	Non-gradient byssal precursor *Mytilus edulis*	AAC33847	0.43
2151	12.9/4.6	9.0/4.6	40	50S Ribosomal protein L7/L12 *C. jejuni*	NP_281664	3×10^{-25}

The mass spectra were recorded on a MALDI mass spectrometer. The amino acid theoretical protein sequences selected in the ORF database by ProteinProspector program were used in the search for proteins with sequence homology of other microbial species listed in the NCBI database using the BLAST program. Theoretical MW/p*I* values were derived from translated ORFs.

PAGE) has no homology with known proteins. In Table 2 experimental p*I* and MW values are also compared with theoretical p*I* and MW values derived from homologues proteins. The major differences between observed and expected molecular mass of proteins concerned proteins with experimental MW below 18 kDa. The major discrepancies in the case of p*I* values concerned proteins with p*I* ranging from 8 to 10. The extreme case is membrane protein Tul4 whose theoretical p*I* value was 9.2 but whose observed value was 4.8. Similar charge and mass variations were described recently for identified proteins of *Mycoplasma genitalium* [53]. Mostly these discrepancies are associated with post-translational modification of proteins. On the other hand, the possibility of incorrectly identified start and end sites of putative ORFs must be also taken into account.

The reference maps with description of identified proteins will be displayed on a web site, which will allow direct access to 2-DE data (http://www.mpiib-berlin.mpg.de/2D-PAGE/microorganisms/index.html).

2.2. Mapping of integral membrane proteins

Membrane proteins due to their interfacial position in cells are involved in wide array of host–microbe interactions such as cell adhesion, protein secretion, modulation of cell signaling and induction of endocytosis [54–56]. However, proteomic analysis of membrane proteins was until recently nearly impossible because of technical problems with their extraction and subsequent solubilization [57]. This was especially true for integral membrane proteins that repeatedly traverse membrane bilayer via α

helices composed of stretches of hydrophobic amino acids. Now the more effective solubilization of membrane proteins is achieved by introduction of new reagents involving thiourea, tributylphosphine (TBP) and amidosulfobetain surfactants SB3-10 or amidosulfobetain-14 (ASB-14) [58]. Molloy et al. [59] described solubilization and successful 2-DE resolution of some *E. coli* outer membrane protein using lysis buffer containing 5 *M* urea, 2 *M* thiourea, 2% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfat (CHAPS), 2% SB3-10 and 2 m*M* TBP. Further progress made in this field of study coincides with the application of a rapid method for isolation of bacterial outer membrane based on the treatment of isolated bacterial membranes with sodium carbonate at high pH [60]. In this way the cytoplasmic proteins and loosely attached membrane proteins are removed and, conversely, the fraction of integral membrane proteins is enriched. The pellets of integral membrane proteins are then re-solubilized in buffer containing the strong solubilizing additives thiourea, ASB-14 and TBP. The suitability of this approach was verified by the analysis of outer membrane proteome of gram-negative bacteria *E. coli* and *C. crescentus* [8,61]. In the case of *E. coli*, 69% of the integral outer membrane proteins predicted for applied 2-DE separation window were identified on a single gel, while as regards the *Caulobacter* study, of the 54 proteins uniquely identified, 41 were outer membrane proteins.

We have applied the same procedure for detection of *F. tularensis* LVS outer membrane proteins. The resulting 2-DE map containing more than 300 spots is shown in Fig. 5. Most proteins occur in several charge variants indicating some type of posttranslational modification. It is statistically estimated that ~20% of all the identified ORFs in bacteria encode putative integral membrane proteins [58]. In the case of *F. tularensis* strain Schu 4 ORFeom, 34% of all putative proteins can be tentatively classified as membrane proteins (see below). This assumption roughly corresponds to our number of detected integral membrane proteins [52]. Up to now, only three proteins have been unambiguously identified in this group of proteins. One of them, denoted TUL4, FRATU 17 kDa, has been previously characterized on a molecular level as a 17-kDa major membrane lipoprotein [62]. The other two identified proteins, 60-kDa chaperonin and hypothetical 23-kDa protein, were originally thought to reside in cytoplasm. However, concerning chaperonins, several recent studies document their extracytoplasmic location in bacteria [63,64]. Membrane-associated localization of bacterial chaperonins was reported in *Salmonella typhimurium* [65], *Mycobacterium leprae* [66], and in several other microbes [67,68]. Membrane forms of chaperonins have different biological functions than their cytoplasmic counterparts. So, while the protection and correct folding of proteins are basic cytoplasmic functions of chaperonins, the membrane chaperonins are implicated in promotion of bacterial adherence and invasiveness [11,69,70]. The Toll-like receptors that are involved in the induction of innate immune response seem to be responsible for interaction with the surface-associated 60-kDa chaperonins [71]. The hypothetical 23-kDa protein is up-regulated during the growth of *F. tularensis* LVS in macrophages or after in vitro exposure of microbes to hydrogen peroxide [72]. This protein showed no homology with any sequence in databases, and therefore, there has been no indication about its biological function. Nevertheless, since its level is increased under stressful conditions, it can be considered as a universal stress protein. Subcellular fractionation and computer analysis of the amino sequence indicated cytoplasmic location of the hypothetical 23-kDa protein [72]. However, in comparison with published results we identified at least three additional charge variants of the hypothetical 23-kDa protein on our 2-DE protein maps. Hence, like in the case of TUL4 where palmitolaytion of protein was decisive for its membrane location, post-translational modification can also play a role in the hypothetical 23-kDa protein membrane attachment.

2.3. Immunoreactive proteins of F. tularensis LVS microbes

F. tularensis infection induces both the cell-mediated and humoral immune response in an infected host. Proteome technology represents a powerful tool for identification of immunologically relevant constituents of *F. tularensis*. Serological proteome analysis, based on a combination of 2-DE and immunoblotting with human tularemic sera, has the potential to reveal new *Francisella* specific markers suitable

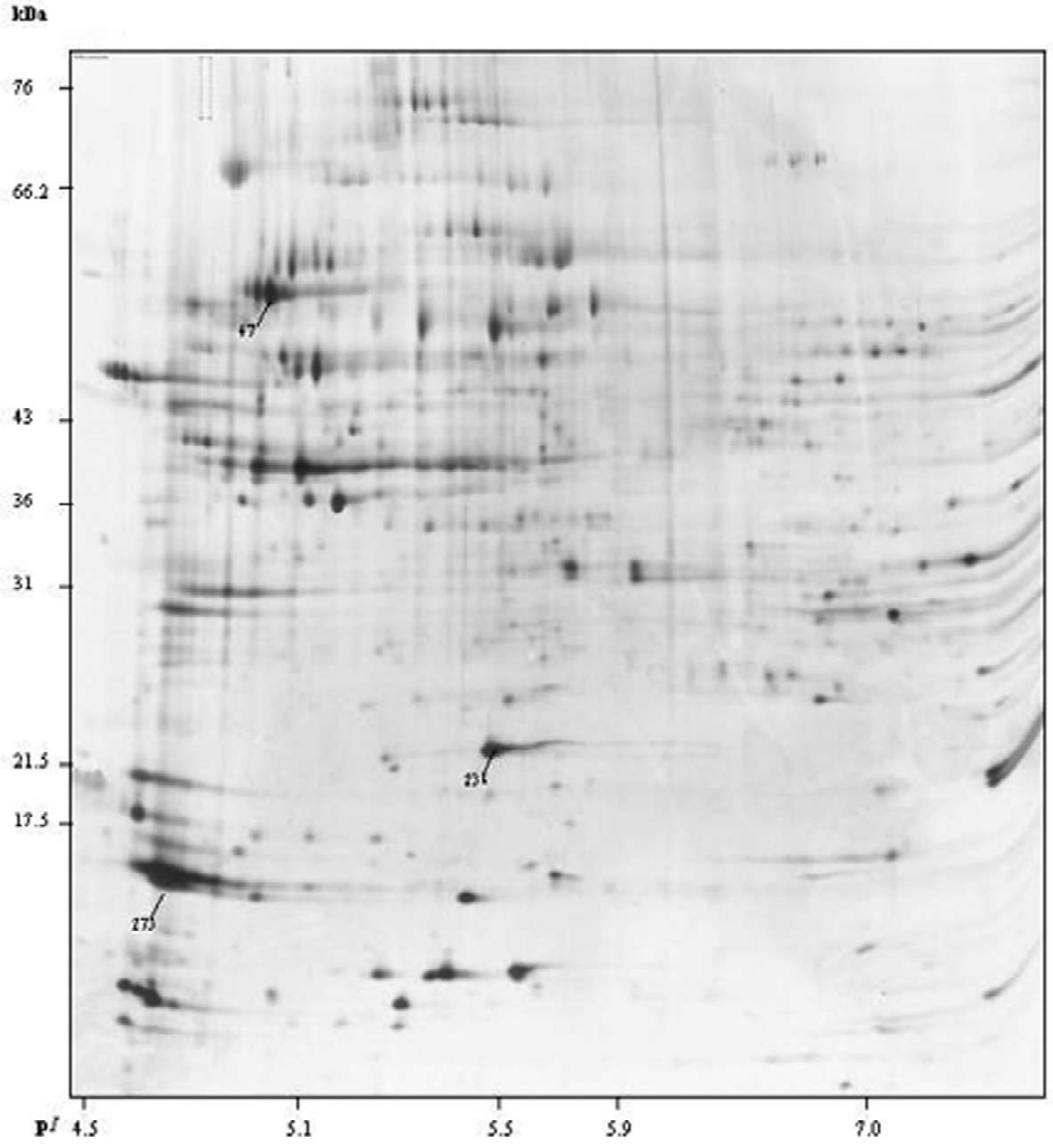

Fig. 5. Silver-stained 2-DE reference map of integral membrane proteins of *F. tularensis* LVS. Isolated membranes were treated with sodium carbonate at high pH. The membrane pellet was solubilized using 5 *M* urea, 2 *M* thiourea, 2% CHAPS, 2% SB3-10, 2 m*M* TBP, 40 m*M* Tris and 2% carrier ampholytes and proteins were resolved by IEF in the pH range 3–10 followed by SDS–PAGE gradient gel (9–16%). The spot numbers indicate identified proteins.

for diagnostic purposes as well as potentially protective antigens useful for the construction of sub-unit vaccine.

Before the introduction of immunoproteomics, complex antigen preparations such as sonicated whole bacteria [73] or outer membrane preparation

containing a variety of proteins [74] were described as immunogens suitable for serodiagnostic tests. Furthermore, *Francisella* lipopolysaccharide (LPS) also proved to be a diagnostically reliable antigen [75]. The reactivity of immune sera collected from human recipients of the live tularemia vaccine appeared to be directed primarily against this outer membrane component of gram-negative bacteria [76,77]. Similarly, a large portion of the specific antibody response in mice infected with LVS bacteria comprised anti-LPS antibodies [78]. Characteristic LPS ladder-like reactivity can be found on immunoblots treated with both mouse and human immune sera (Fig. 6). As can be seen from this figure, the mouse antibody response exhibiting pronounced reactivity in the low molecular weight region differed clearly from the antibody response elicited in infected human beings (unpublished results).

Besides the lipopolysaccharide, other components of *F. tularensis* outer membrane have been described as immunogens as well. Especially, membrane proteins are potent inducers of specific T cell response in *F. tularensis* primed humans [79]. One of them, a 17-kDa major membrane lipoprotein denoted TUL4, induced a strong cellular as well as antibody immune response in both vaccinated and naturally infected individuals [80]. Protective immunity against LVS infection has been demonstrated in mice as a consequence of immunization with recombinant *S. typhimurium* strain expressing TUL4 [81]. A similar protective effect was found after immunization with immunostimulating complexes into which TUL4 had been incorporated [82]. As already mentioned, systemic mapping of the immunoreactive *F. tularensis* LVS integral membrane proteins, prepared by the carbonate extraction method according to Molloy and coworkers [8], has been started in our laboratory. Using three seropositive tularemic sera, ~50 immunoreactive protein spots were detected on 2-D immunoblots [83]. Among them, TUL4 provided strong reaction with all tested sera (Fig. 7). The dnaK and 60-kDa chaperonin are other targets of the humoral antibody response to *F. tularensis*. The immunoreactivity of these two bacterial stress proteins has been found in sera from naturally infected individuals, as well as in volunteers vaccinated with *F. tularensis* LVS. On the other hand, weak cross-reactivity was also disclosed in some control sera taken from blood donors [84]. This is entirely consistent with our own recently published observations (Table 3) [83].

Furthermore, antibody activity against 10-kDa chaperonin has been demonstrated in this study (Fig. 7). The four charge and mass variants of 10-kDa chaperonin occurred on the 2-DE map of *F. tularensis* LVS and an additional two 10-kDa chaperonin spots were then identified on the 2-DE map of *F. tularensis* strain 176 (Table 1, Figs. 1 and 4). The existence of such multiple bacterial variants of 10-kDa chaperonin has already been described in a proteome study of *M. tuberculosis* [85]. According to the obtained results, 10-kDa chaperonin seems to be a promising candidate for *Francisella* specific marker because of its specificity and immunogenicity (Table 3). Immune reactions with at least one variant of the protein were found in four out of nine individuals undergoing tularemia, whereas no reaction was disclosed in control sera.

The list of all immunoreactive proteins identified to date by means of serological proteome analysis is shown in Table 3. In total, 22 immunogenic spots have been successfully identified and 11 of them have provided specific reactions only with sera from tularemia patients. In order to complete the reference map of immunoreactive proteins, several other strongly immunostained spots remain to be identified, especially those on 2-D map of carbonate extracted integral membrane proteins.

2.4. F. tularensis protein profiling under conditions mimicking a hostile intracellular environment

When bacteria enter the host organism they undergo phenotypic modulation controlled by the coordinated gene expression in order to survive harsh conditions and hostile environmental conditions [86]. Moreover, intracellular parasites, which invade and multiply within cells, specialized to their ingestion and killing, must resist intracellular defensive mechanisms of both oxidative and non-oxidative origin. The oxygen dependent mechanisms rely on the generation of reactive oxygen metabolites, such as superoxide anion, hydroxyl radicals, hypochlorite ions, hydrogen peroxide, and singlet oxygen inside the phagosome. The acidification of intraphagosomal

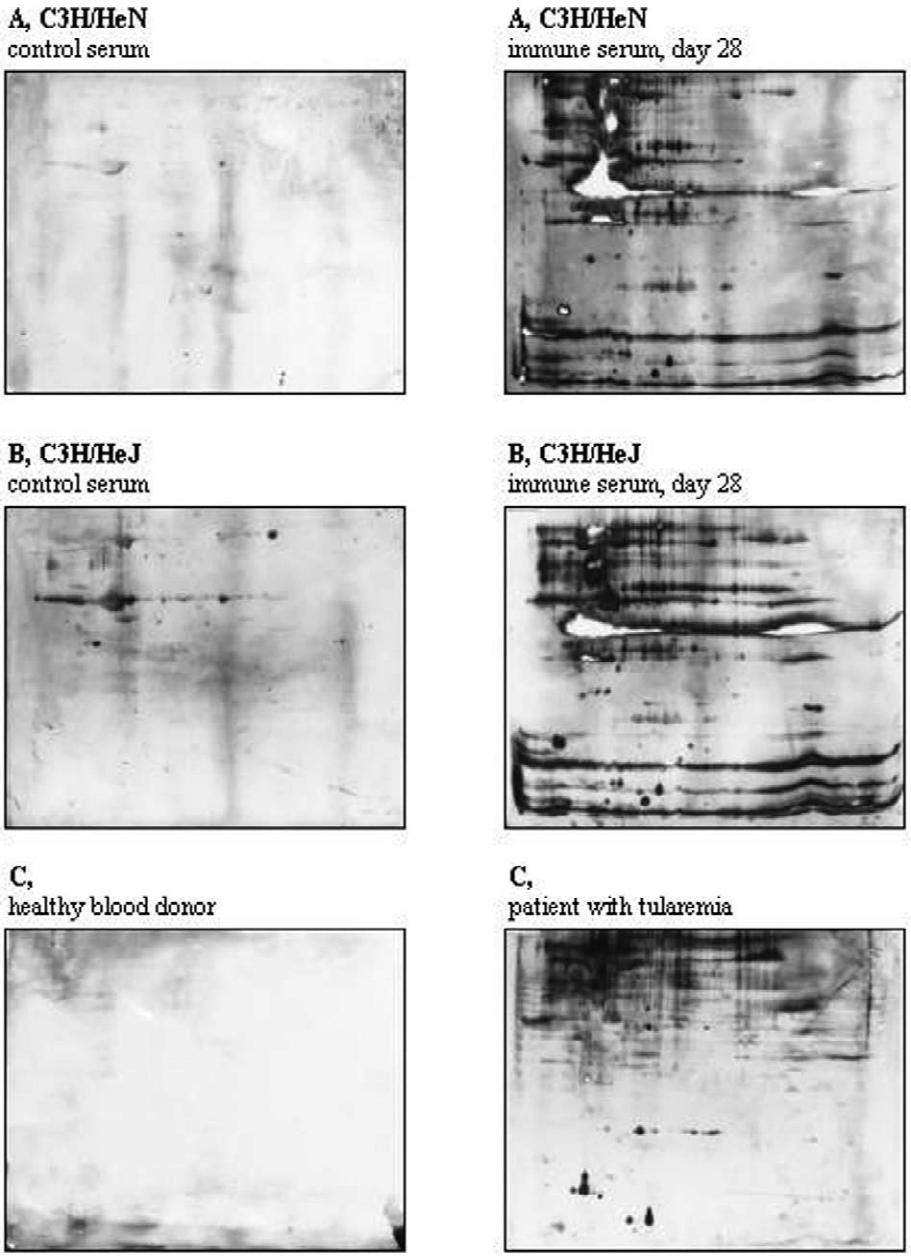

Fig. 6. 2-D immunoblotting pattern of specific antibody response to *F. tularensis* infection. The immune sera were collected from C3H/HeN (*Lps*^{n}) mice (A), and from their congenic C3H/HeJ (*Lps*^{d}) counterparts (B), 28 days after *F. tularensis* LVS challenge. Mice were inoculated s.c. with a dose of 100 microbes. Non-immune control sera originated from mice treated only with saline. For comparison, the representative human immunoreactivity pattern is shown (C). Human tularemic serum was obtained from a patient with natural infection. As a control, serum from a healthy blood donor without a history of tularemia was used. The whole cell bacterial lysate of *F. tularensis* LVS was separated on pH 3–10 IPG strips, followed by 9–16% SDS–PAGE. All the sera were diluted 1:100. As a secondary antibody anti-IgG, IgA, and IgM peroxidase conjugate was used. Reactions were visualized with enhanced chemiluminiscence based kit.

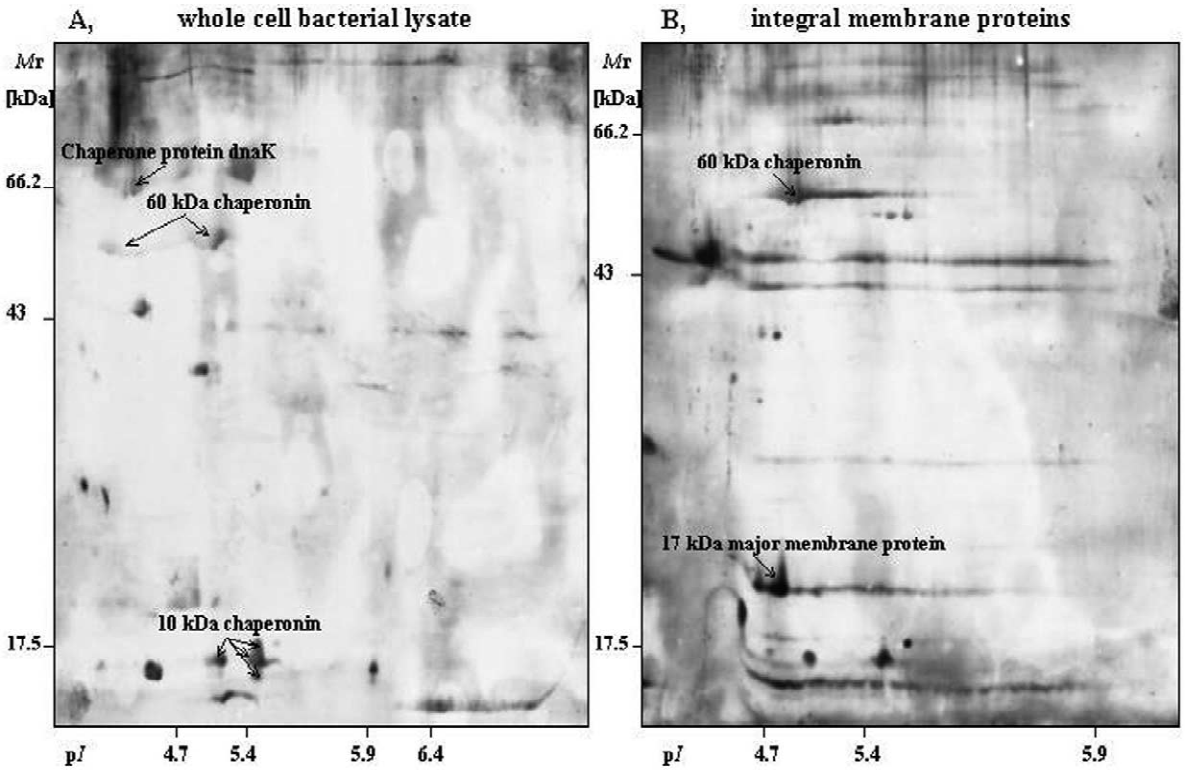

Fig. 7. An example of a 2-D immunoblotting pattern of antigens recognized by human tularemic serum. Whole cell bacterial lysate *F. tularensis* LVS (A) and integral membrane proteins, isolated by the carbonate extraction method according to Molloy and coworkers [8] (B), were resolved on pH 3–10 IPG strips with subsequent 9–16% SDS–PAGE [84]. Both blotting membranes were treated with the same tularemic serum. As a secondary antibody anti-IgG, IgA, and IgM peroxidase conjugate was used. Reactions were visualized with enhanced chemiluminiscence based kit.

space, release of hydrolytic enzymes and small defensine molecules, and deprivation of nutrients are then examples of cellular oxygen-independent defensive potential. Actually each stress factor induces a specific set of proteins. Therefore, the proteomic analysis of changes in bacterial gene expression under the influence of real intracellular conditions or conditions mimicking an adverse intracellular milieu offers the unique possibility of identifying molecules responsible for virulence and pathogenicity of intracellular bacterial pathogens [87].

To determine the pattern of bacterial gene expression in terms of defined responses to conditions relevant to the intracellular environment, several in vitro systems were exploited. The cultivation of bacteria under selected stress conditions, e.g. low or high concentrations of iron; extreme temperatures, acidic pH, or oxidative stress, were used for the mapping of molecules significant for microbial pathogenicity. The most sophisticated system for this type of study seems to be the radiolabeling of bacterial proteins after in vitro ingestion of microbes by phagocytic cells. The utilization of this complex in vitro system reflects the facts documenting that the bacterial response to intracellular growth is not simply the sum of responses to individual stress conditions mentioned above [21].

Currently there is little data concerning the modulation of *F. tularensis* gene expression in the course of microbe–host cell interaction. Analysis of protein synthesis of *F. tularensis* LVS growing intracellularly in the macrophage-like murine J774 macrophages demonstrated induction of only a few proteins [72]. One-dimensional electrophoresis of bacteria pulse-

Table 3
The list of identified *F. tularensis* LVS immunoreactive proteins

Protein				2-D immunoblotting reactivity		
Protein name Organism	Accession No.	Spot No.	MW (kDa)/p*I*, measured	Tularemia	Borreliosis	Blood donors
Whole-cell antigen *F. tularensis* LVS IEF pH 3–10 9–16% SDS–PAGE						
Chaperone protein dnaK *F. tularensis*	P48205	120	69.2/4.9	+++	+/−	+
60-kDa Chaperonin *F. tularensis*	P94798	214	55.6/4.9	+++	+	–
Elongation factor TU *S. typhimurium*	P21694	340	47.8/4.6	+++	+++	–
Glycine-cleavage system protein T1 *P. aeruginosa*	G82994	488	40.3/5.9	+++	–	–
Oxidoreductase *V. cholerae*	G82383	753	25.7/6.3	+	–	–
Hypothetical protein *P. falciparum*	CAB38995	755	25.6/5.3	+	–	–
Hypothetical 23-kDa protein *F. tularensis*	CAA70085	790	23.3/5.4	+/−	+	+/−
Biotin carboxyl carrier protein *P. aeruginosa*	P37799	889	13.7/4.9	+++	+/−	–
50S Ribosomal protein L9 *P. aeruginosa*	F83029	893	13.3/5.4	–	+	+
Probable bacterioferritin *P. aeruginosa*	B83036	918	10.3/5.4	+++	+/−	–
10-kDa Chaperonin *F. tularensis*	P94797	920	10.3/5.2	++	–	–
10-kDa Chaperonin *F. tularensis*	P94797	931	9.7/4.9	+++	–	–
50S Ribosomal protein *C. jejuni*	H81392	933	9.6/4.6	+++	++	+++
10-kDa Chaperonin *F. tularensis*	P94797	939	9.3/5.3	+++	–	–
3-Dehydroquinase *Buchnera* sp.	P57479	949	9.4/6.0	++	–	–
10-kDa Chaperonin *F. tularensis*	P94797	962	8.6/5.3	++	–	–
Whole-cell antigen *F. tularensis* LVS IEF pH 6–11 9–16% SDS–PAGE						
Histone-like protein HU form B *P. aeruginosa*	AF345628	478	14.5/9.1	++	+/−	–
Integral membrane proteins *F. tularensis* LVS IEF pH 3–10 9–16% SDS–PAGE						
60-kDa Chaperonin *F. tularensis*	P94798	47	57.4/5.0	+++	–	–
17-kDa Major membrane protein (precursor) *F. tularensis*	P18149	275	13.3/4.8	+++	+	–

Human tularemic sera originating from nine seropositive patients were used to screen for the immunoreactive antigens of *F. tularensis* by the means of 2-D immunoblotting [83]. As controls, sera from two blood donors and three individuals with Lyme disease were used. Control sera were anti-*F. tularensis* negative according to microagglutination, but displayed some immunoreactivity with *F. tularensis* antigens on 1-D immunoblots. Maximum intensity of immunostaining found on 2-D immunoblots is shown in the table. The staining intensity was evaluated visually and classified from − to +++. Either MALDI–TOF-MS (whole cell lysate), or LC–MS–MS (integral membrane proteins) was used for identification. Both the *F. tularensis* proteins included in the NCBI or SWISSPROT protein databases, as well as sequence homologues originating from other bacteria, are presented.

labeled at 24 h of intracellular growth revealed the increased synthesis of four proteins, of which only one protein, hypothetical 23-kDa protein, was shown to be also induced using 2-DE gels. Surprisingly, with the exception of dnaK, the 60- and 10-kDa chaperonins were only marginally or not at all induced. Upregulation of the hypothetical 23-kDa protein was also found after exposure of *F. tularensis* LVS to hydrogen peroxide. In contrast, the temperature shift to 42 °C that led to the increased expression of at least 15 proteins, involving dnaK, 60- and 10-kDa chaperonins, did not influence the hypothetical 23-kDa protein level significantly [72].

3. Comparative analysis of *F. tularensis* type A and B strains

A unique set of phenotypic characteristics, including morphology and biochemical markers, made isolates of the *Francisella* genus readily distinguishable. However, within the genus and particularly within species *F. tularensis*, the strain discrimination is not performed conveniently. Previous genetic studies have demonstrated that the four subspecies of *F. tularensis*, despite showing marked variations in their virulence for mammals and originating from different regions throughout the northern hemisphere, display a very close phylogenetic relationship. Due to the contagiousness of *Francisella* isolates only limited work has been performed to develop typing methods based on cultivation. Recent development in genetic analysis made novel strategies based on PCR discrimination available. Johansson and his group [88] tested three PCR methods which all generated similar subspecies specific patterns, however, the discriminatory indices varied within the range, which is not sufficient to distinguish individual strains. They proposed the combination of one of these methods and PCR employing subspecies-specific primers as a suitable, rapid and relatively simple strategy for discrimination of *Francisella* species and subspecies. Identification of subspecies specific markers using whole-genome DNA micro array and subsequent clustering reveals similar data regarding subspecies distribution [34]. Both results suggest that Japanese strains sorted to subsp. *holarctica* represent a separate subspecies and that type strain ATCC6223 differ from other strains, which authors explain by genetic changes during storage and passages in small animals. The DNA micro array study shows an almost identical pattern of subsp. *mediaasiatica* and subsp. *tularensis* which contradicts current strain designation. PCR study does not support this result.

A thorough comparative proteome analysis of different strains is under way. The 19 strains included so far in the study belong to three subspecies of *F. tularensis—tularensis*, *holarctica* and *mediaasiatica* (Table 4). The 2-DE map of whole cell lysate of each strain has been performed on a wide gradient of pH 3–10 (IPG strips) and spot patterns of all gels have been compared using Melanie III software. With preliminary comparison, an automatic correspondence analysis (Melanie III) has been performed after landmark identification and gel alignment. Correspondence analysis allows representation of series of gels, each having a large number of spots, in a factorial space of reduced dimension. Gels appear as points on the planar graphic plot and the distance between points corresponds to their similarity [89]. This analysis applied to 19 images of gels revealed a distinct pattern of distribution. Whilst all *holarctica* strains were grouped together, *tularensis* strains scatter more dramatically, however, they were clearly distinguished from *holarctica* strains (unpublished results). The most diverse locations exhibit *tularensis* strains harboring resistance to antibiotics (FAM SR which is streptomycin resistant, and TTCR 6-4-1 which is tetracycline resistant) and ATCC *tularensis* type strain ATCC6223. The separation of ATCC variant is in accordance with the results from both genetic studies mentioned above and indicates the loss of some characteristics typical for wild *tularensis* isolates. The distribution of antibiotic resistant strains suggests large changes in protein profiles associated with the induction of antibiotic resistance and presents an interesting task for further analyses. The separate grouping of two *mediaasiatica* strains with respect to both *tularensis* and *holarctica* subsp. supports their distinct subspecies annotation, although this result needs further evaluation.

For the purpose of comparative proteomic study of subsp. *tularensis* and *holarctica*, the strain selection reflected correspondence analysis distribution (the

Table 4
F. tularensis strains included in comparative analysis

FSC No.[a]	Subspecies	Origin	Other names	Selected[b]
13	*tularensis*	(from Eigelsbach)	FAM standard	Yes
14	*tularensis*	Strepto resistant FAM-variant	FAM SR	No
41	*tularensis*	Tick, British Columbia, Canada, 1935	Vavenby	Yes
43	*tularensis*	Human ulcer, 1941, Ohio	Schu	Yes
122	*tularensis*	Ttc resistant Schu-strain	TTCR 6-4-1	No
199	*tularensis*	Mite, Slovakia, 1988	SE-221-38	Yes
230	*tularensis*	Human lymph node, 1920, Utah	ACC 6223	No
237	*tularensis*	Human ulcer, 1941, Ohio	Schu-S4	Yes
17	*holarctica*	Human lymph node, 1926, Japan	Jap-S2	No
35	*holarctica*	Beaver 1976, Hamilton, Montana	B423 A	Yes
69	*holarctica*	T7 passaged in animals, more virulent	SVA T7K	No
74	*holarctica*	Hare, Sweden, 1974	SVA T7	Yes
124	*holarctica*	Water, 1990, Odessa region, Ukraine	14588	No
155	*holarctica*	Live Vaccine Strain, LVS, Russia	ATCC 29684	No
200	*holarctica*	Human, 1998, Ljusdal, Sweden	Rem nr 3001	Yes
247	*holarctica*	Human, 1993, Vosges, France	SVA T20	Yes
257	*holarctica*	Tick, Moscow area, 1949, Russia	503/840	Yes
147	*mediaasiatica*	Miday gerbil, 1965, Kazakhstan	543	–
148	*mediaasiatica*	Ticks, 1982, Central Asia	240	–

The 2-DE map of whole cell lysate on wide range IPG strip (pH 3–10) of each strain has been prepared and correspondence analysis performed. Subsequently, strains for *holarctica* versus *tularensis* comparison have been selected based on 2-DE map similarity within subspecies designation.

[a] Francisella Strain Collection number at SDRA.

[b] Strains selected for *holarctica* versus *tularensis* comparison.

most related strains were selected). Five strains, each represented by one gel, of subsp. *tularensis* and five strains, each represented by one gel, of subsp. *holarctica* were included (Table 4). Out of the total of 69 protein spot differences, 30 were unique to *tularensis* strains and 39 to *holarctica* strains. The identification of the specific protein by peptide mass fingerprinting and other mass spectrometry based methods is ongoing and, currently, seven differentially expressed proteins have been identified (Table 5). Two proteins specific for subsp. *tularensis*, the most basic variant of hypothetical 23-kDa protein and acid phosphatase (Acp), have been identified previously [90]. As described by Golovliov et al. [72], hypothetical 23-kDa protein was the most upregulated protein during intracellular growth of *F. tularensis* vaccine strain LVS in macrophages, pinpointing this protein as one of the possible virulence factors. Acid phosphatase specificity corresponds with the common idea that this enzyme belongs to a group of virulence factors of intracellular pathogens. Nevertheless, some unique features in the structure and substrate specificity of tularemic Acp as compared to other burst-inhibiting Acps makes it difficult to fit this protein into currently recognized classes of acid phosphatases [91] and thus makes explanation of the mechanism leading to increased pathogenicity unclear. Out of 39 proteins specific for *holarctica* strains, five spots have been identified so far. Among them the expression of bacterioferritin (Fig. 8) exhibited the most striking difference [23]. Recently published work aimed at comparative protein profiling of vaccine and virulent strains of *Brucella melitensis* [92] described a similar finding with characteristic expression of bacterioferritin in vaccine strain. In this study, not only bacterioferritin but also two other proteins involved in iron transport and utilization have been upregulated in vaccine strain. Deprivation of iron is an important factor of host defense against pathogenic bacteria and the battle for iron is one of the most important microbe–host interactions [93].

Table 5
The list of identified proteins with unique expression in *holarctica* or *tularensis* subspecies

Protein name Organism	Accession No.	MW (kDa)/p*I*, theoretical	MW (kDa)/p*I*, measured	Sequence coverage, %
Specific for holarctica				
Probable bacterioferritin *P. aeruginosa*	B83036	18.5/5.3	10.3/5.4	40
Probable sigma (54) modulation protein *P. putida*	P15592	11.2/5.9	7.8/6.3	46
Putative succinyl-CoA synthetase alpha subunit *Neisseria meningitidis* Z2491	NP-283922	30.1/6.1	36.1/5.9	21
Nucleoside diphosphate kinase *Xylella fastidiosa* 9a5c	NP-297748	15.5/5.9	15.2/5.9	31
Cationic 19-kDa outer membrane protein precursor *Yersinia*	P315119	18.8/7.7	14.4/6.4	34
Specific for tularensis				
Acid phosphatase *F. tularensis*	AAB06624	57.6/5.9	59.0/6.0	23
Hypothetical 23-kDa protein *F. tularensis*	CAA70085	22.1/5.7	23.0/6.0	30

Specific spots for each subspecies have been selected based on 2-DE map comparison of selected strains. Out of 69 specific spots, seven proteins, mentioned in the table, have been identified so far by comparing spot position to position of proteins already identified by peptide mass fingerprinting in 2-DE database of *F. tularensis* LVS (subsp. *holarctica*) and *F. tularensis* 176 (subsp. *tularensis*).

4. Construction of virtual and real proteome of *F. tularensis*—from genome to proteome

In order to verify and extend our knowledge of newly identified *F. tularensis* proteins, we have analyzed translated ORF of each identified protein by algorithms providing information about functional and topological protein classification. The computing working on the basis of sequence similarity is able to discriminate between soluble and membrane proteins

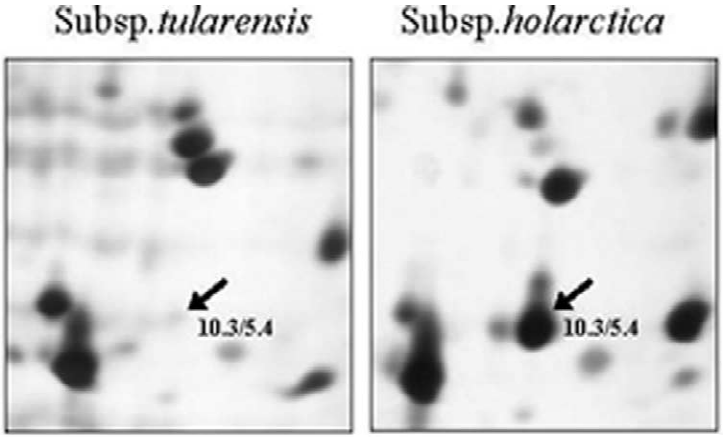

Fig. 8. Enlargement of a section of the 2-DE map showing different expressions of bacterioferritin (arrow) between subsp. *tularensis* (FSC 199) and *holarctica* (FCS 74). Silver stained 2-DE gels, pH 3–10, 9–16% SDS–PAGE.

(SOSUI), to predict intracellular topology (PSORT) of proteins and to determine the tentative function of proteins (COG algorithm). The data obtained from this analysis are being used for the formation of a so-called ''virtual proteome'' of *F. tularensis* microbe.

A software system, SOSUI, was developed for discriminating between soluble and membrane proteins together with the prediction of transmembrane helices [94]. The performance of the system was 99% accurate for discrimination between two types of proteins and 96% for prediction of transmembrane helix [95]. Preliminary analysis of *F. tularensis* ORFeom by the SOSUI system predicted that 34% of all putative proteins could be located in bacterial membrane. However, the data should be taken with caution because prediction of membrane location with reasonable confidence necessitates the evaluation of additional criteria such as sequence similarities to other membrane proteins and the existence of signal sequence for translocation of protein to outer membrane. For this purpose the localization of proteins was also verified by the PSORT algorithm which was developed to predict protein topology inside gram-positive and gram-negative bacteria, yeast, animal and plant cells. The predicted candidate localization-sites for gram-negative bacterium are bacterial outer membrane, bacterial periplasmic space, bacterial inner membrane and bacterial cytoplasm. PSORT first predicts the presence of signal sequences by the method of McGeoch [96] modified by Nakai and Kanehisa [97]. Further, PSORT applies von Heijne's method [98] of signal sequence recognition. The next parameters that PSORT calculates are transmembrane segments and lipoproteins according the methods published earlier [99,100]. The functional classification of tentative proteins was obtained by applying the Clusters of Orthologous Groups of proteins (COGs) algorithm. The COG database has been developed as a phylogenetic classification of proteins from complete genomes [101]. The COGs have been classified into 17 broad functional categories, including a class for which a general function prediction, usually that of biochemical activity, was feasible and a class of uncharacterized COGs. In a strict sense, the COGNITOR program which accompanies the COG database and assigns new proteins [102] was used for prediction of identified *F. tularensis* proteins function. The analyzed data covering both identified proteins with known function and hypothetical proteins are shown in Table 6. As can be seen from this table, proteins identified by mass spectrometry as enzymes participating in the metabolism of amino acids, carbohydrates, lipids, nucleotides or energy production, have been sorted into the COG category that corresponds with their assigned function. This further confirms the reliability of the identification approach. The same is true for chaperones, transcription and translation factors. Topological prediction was, for all the above-mentioned functional groups, besides chain A, structure of inorganic pyrophosphatase, determined as bacterial cytoplasm protein. The function of remaining identified proteins listed in Table 6 was unknown. Two proteins, peptidoglycan-associated lipoprotein Pal and IMI-1, were classified by COG in the COG-M category, i.e. cell envelope biogenesis, outer membrane which is also in agreement with their predicted topological location (bacterial inner membrane proteins containing signal peptides). The classification of hypothetical transmembrane protein was COG-P-inorganic ion transport and metabolism. Both algorithms SOSUI and PSORT predicted its membrane position. For five protein classifications COG-S-function was unknown or no related COG were found. For the latter, the group includes two membrane proteins not described before and the already mentioned TUL4 and hypothetical 23-kDa proteins. The topography prediction of TUL4 documents the necessity to combine SOSUI and PSORT algorithms. As was stated, TUL4 is integral membrane protein anchored in membrane via palmitic acid. Because of the lack of transmembrane domains, SOSUI recognized it as soluble protein, however, by the PSORT algorithm it was designated as an outer membrane protein.

5. Conclusions

The current proteomic approach still suffers from several technical limitations that do not allow comprehensive analysis of the expression of many important classes of proteins to be performed. However, despite this, even initial bacterial proteome analyses are already able to yield important data contributing to a more detailed understanding of the pathogenesis of infectious diseases. This further

Table 6
Prediction of membrane association using programs SOSUI and PSORT, and functional assignment of genes based on placing ORF in COG category

Protein name (homologue)	Structure–function relationship (COG-code)	Topography prediction		Signal peptide
		SOSUI	PSORT	
NAD-specific glutamate dehydrogenase	Amino acid transport and metabolism (COG-E)	Soluble protein	Bacterial cytoplasm protein	No
Glutamine synthetase, chloroplast precursor	Amino acid transport and metabolism (COG-E)	Soluble protein	Bacterial cytoplasm protein	No
Phosphoglycerate kinase	Carbohydrate transport and metabolism (COG-G)	Soluble protein	Bacterial cytoplasm protein	No
Fructose-bisphosphate aldolase	Carbohydrate transport and metabolism (COG-G)	Soluble protein	Bacterial cytoplasm protein	No
Peptidoglycan-associated lipoprotein Pal	Cell envelope biogenesis, outer membrane (COG-M)	Soluble protein	Bacterial inner membrane protein	Yes
IMI-1	Cell envelope biogenesis, outer membrane (COG-M)	Soluble protein	Bacterial inner membrane protein	Yes
Citrate synthase	Energy production and conversion (COG-C)	Soluble protein	Bacterial cytoplasm protein	No
Chain A, structure of inorganic pyrophosphatase	Energy production and conversion (COG-C)	Soluble protein	Bacterial inner membrane protein	No
Sigma cross-reacting protein 27A	Function unknown (COG-S)	Soluble protein	Bacterial inner membrane protein	No
Macrophage growth locus B	General function prediction only (COG-R)	Soluble protein	Bacterial cytoplasm protein	No
Hypothetical transmembrane protein	Inorganic ion transport and metabolism (COG-P)	Membrane protein	Bacterial inner membrane protein	Yes
Probable bacterioferritin	Inorganic ion transport and metabolism (COG-P)	Soluble protein	Bacterial cytoplasm protein	Yes
FabD	Lipid metabolism (COG-I)	Soluble protein	Bacterial cytoplasm protein	No
Transport protein homolog	No related COG	Membrane protein	Bacterial inner membrane protein	Yes
Non-gradient byssal precursor	No related COG	Membrane protein	Bacterial inner membrane protein	Yes
Hypothetical 23-kDa protein	No related COG	Soluble protein	Bacterial cytoplasm protein	No
TUL4, FRATU 17 kDa	No related COG	Soluble protein	Bacterial outer membrane protein	Yes
Thymidylate kinase	Nucleotide transport and metabolism (COG-F)	Soluble protein	Bacterial cytoplasm protein	No
Probable thymidylate synthase	Nucleotide transport and metabolism (COG-F)	Soluble protein	Bacterial cytoplasm protein	No
Chaperone protein dnaK	Posttranslation modification, protein turnover, chaperons (COG-O)	Soluble protein	Bacterial cytoplasm protein	No
60-kDa Chaperonin	Posttranslation modification, protein turnover, chaperons (COG-O)	Soluble protein	Bacterial cytoplasm protein	No
10-kDa Chaperonin	Posttranslation modification, protein turnover, chaperons (COG-O)	Soluble protein	Bacterial cytoplasm protein	No
Transcription elongation factor	Transcription (COG-K)	Soluble protein	Bacterial cytoplasm protein	No
50S Ribosomal protein L9	Translation, ribosomal structure and biogenesis (COG-J)	Soluble protein	Bacterial cytoplasm protein	No

encourages rapid progress in improvements of technologies for protein analysis that should in combination with other complementary techniques, such as molecular biology, enable complex microbial systems to be studied in their entirety.

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