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Identification of Potentially Involved Proteins in Levofloxacin Resistance Mechanisms in *Coxiella burnetii*

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The etiological agent of Q fever, *Coxiella burnetii*, is an obligate intracellular bacterium that multiplies within a phagosome-like parasitophorous vacuole. Fluoroquinolones have been used as an alternative therapy for Q fever. Resistance to fluoroquinolones can arise via several mechanisms utilized by pathogens to avoid killing. Until today, genome-based studies have shown that the main mechanism of *C. burnetii* to resist inhibition by fluoroquinolones is based on mutations in quinolone-resistance-determining region (QRDR). In this study, in a broader search at the protein level for *C. burnetii* mechanisms that confer resistance to fluoroquinolones, the proteomes of *in vitro* developed fluoroquinolone resistant bacteria and susceptible bacteria were compared using the MS-driven combined fractional diagonal chromatography (COFRADIC) proteomics technique. Quantitative comparison of the 381 proteins identified in both strains indicated the different expression of 15 bacterial proteins. These proteins are involved in different cellular processes indicating that the antibiotic resistance mechanism of the bacterium is a multifaceted process.

Keywords: *Coxiella burnetti* • antibiotic resistance • fluoroquinolones • levofloxacin • MS-driven proteomics • COFRADIC

Introduction

The etiological agent of Q fever, *Coxiella burnetii*, is an obligate intracellular bacterium that multiplies within vacuoles of phagolysosomal origin. Two major forms of the disease are known: acute and chronic. Although the acute form is usually manifested as a self-limiting febrile illness with pneumonia and hepatitis being some of its complications, the chronic form is a severe disease with a mortality rate of 5% which if left untreated ranges from 25% up to 60%,[1] during which the predominant clinical features are blood culture-negative endocarditis, infections of vascular aneurysms or prosthesis and osteomyelitis.[2] The current recommended treatment for acute Q fever is doxycycline.[3] Fluoroquinolones are considered to be a reliable alternative and have been advocated for patients with Q fever meningoencephalitis, because they penetrate the cerebrospinal fluid.[3] On the other hand, treating the chronic form of Q fever still is a therapeutic problem. The recommended regimen is again the combination of doxycycline with hydroxychloroquine with the optimal duration of therapy still remaining unknown and ranging from 18 months to indefinite administration of antibiotics. Of interest is that *C. burnetii* has been recovered from cardiac valve tissue removed from a patient with Q fever endocarditis despite 4 years of antibiotic therapy with tetracycline.[4] Furthermore, *C. burnetii* strains resistant to doxycycline (MIC 8 mg/mL) have been isolated from patients with Q fever endocarditis.[5] Unfortunately, neither doxycycline nor quinolones are bactericidal *in vitro*.[6,7] Clinical data support the lack of *in vitro* rickettsiacidal activity of fluoroquinolones. Relapses after discontinuation of antibiotic therapy remain frequent in chronic Q fever patients treated with the combination of a fluoroquinolone plus doxycycline or rifampin.[8]

Quinolones are inhibitors of the essential bacterial enzymes DNA gyrase and DNA topoisomerase IV.[9] These are large and complex enzymes, each composed of two subunits. The 97 kDa gyrA and the 90 kDa gyrB proteins encoded by the *gyrA* and *gyrB* gene, respectively, are the subunits of the DNA gyrase, whereas, the corresponding subunits of topoisomerase IV are ParC (75 kDa) and ParE (70 kDa). Both enzymes work together in DNA replication, transcription, recombination and repair by rapidly breaking both strands of double-stranded DNA and passing a second DNA double helix through the break which is then released.[10] Quinolones block this reaction by trapping gyrase or topoisomerase IV as a drug-enzyme-DNA complex, with the consequent discharge of fatal double-stranded DNA breaks.[11]

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Resistance to quinolones has been a problem ever since nalidixic acid was introduced into clinical medicine more than 40 years ago. Generally, three mechanisms of resistance to quinolones are currently recognized: mutations that alter the drug targets, mutations that reduce drug accumulation and plasmids that protect cells from the lethal effects of quinolones.[12] In gram-negative bacteria, gyrase is more susceptible than topoisomerase IV to inhibition by quinolones, thus resistance mutations occur first in gyrA. Resistance in gram-negative bacteria involves amino acid substitutions in a region of the gyrA subunit termed "the quinolone-resistance-determining region-QRDR" which is located on the DNA-binding surface of the enzyme.[13] In the case of *Escherichia coli* DNA gyrase, the QRDR includes amino acids between positions 51 and 106.[14] After a first step mutation has reduced the susceptibility of DNA gyrase, further mutations in *gyrA* or mutations in *gyrB* or *parC* can further enhance resistance.[15]

Gram-negative bacteria can regulate membrane permeability by altering the expression of outer membrane porin proteins that form channels for passive diffusion such as outer membrane proteins OmpF and OmpC in *E. coli*[16] providing increased resistance to antibiotics. In addition, the energy-dependent efflux systems some of which are expressed constitutively and others which are controlled by global regulatory systems or are inducible by mutation, can confer elevated resistance to quinolones and xenobiotics in general by prohibiting the antibiotic from reaching its target.[17] In *E. coli*, for instance, the AcrAB-TolC efflux pump plays a major role in quinolone efflux.[18] Finally, plasmids can also directly produce resistance to quinolones. Plasmid-mediated resistance to quinolones, long-thought not to exist, was discovered in a clinical isolate of *K. pneumoniae* from Alabama that could transfer low-level resistance to quinolones to *E. coli* and other gram-negative bacteria.[19]

Little is known about the mechanisms of resistance to quinolones in *C. burnetii*. Musso and his co-workers compared the sequences of QRDRs from susceptible and resistant *C. burnetii* isolates revealing a nucleotide mutation in the resistant strain leading to an amino acid substitution of Gly instead of Glu at position 87 of GyrA suggesting that high-level resistance to quinolones is associated with this mutation.[20] Based on these data, four years later a PCR-RFLP assay was used for the diagnosis of quinolone resistant strains which confirmed the findings of the study by Musso and his co-workers.[21] Two years later a comparison between resistant and pefloxacin susceptible *C. burnetii* strains indicated a difference (lower concentration in the resistant strains) in the intracellular accumulation of the antibiotic suggesting higher penetrability of pefloxacin into quinolone-susceptible *C. burnetii* strains.[7] This fact was suggested to occur due to decreased membrane permeability as a result from the decreased expression of the *C. burnetii*-resistant outer membrane porin proteins. However, there were no differences observed when the outer membrane proteins of the two strains were compared. In a more recent study[22] the sequences of QRDRs from susceptible and *in vitro* developed-resistant *C. burnetii* strains to a second generation fluoroquinolone were compared revealing several nucleotide mutations supporting and adding to the findings of the research by Musso.[20]

Having undeniably established the existence of mutations that alter the drug targets as a mechanism for fluoroquinolone resistance in *C. burnetii*, in this study, in a more extensive quest of the molecular mechanisms that the intracellular pathogen implements to resist inhibition by quinolones, we quantitatively compared proteomes of the reference strain *C. burnetii* Nine Mile RSA 439 that developed *in vitro* resistance to levofloxacin to its respective susceptible strain using COFRADIC.

Materials and Methods

***C. burnetii* Culture and *in vitro* Development of Fluoroquinolone Resistance.** *C. burnetii* Nine Mile (RSA439) phase II reference strain for Q fever was propagated in confluent African Green Monkey kidney fibroblasts (Vero; ATCC no. CCL-81) in 225 cm^2 angled neck flasks (Corning Inc., U.S.A.). Infected cells were cultured in minimum essential medium (MEM; Gibco Laboratories) supplemented with 4% fetal bovine serum (FBS; Gibco Laboratories and 2 mM L-glutamine (Gibco Laboratories)) at 35 °C in a 5% CO_2 incubator. Infection was monitored by Gimenez staining.[23] The levofloxacin (LVX)-resistant *C. burnetii* strain NMres (MIC: 32 mg/L) was derived from the LVX susceptible reference strain NM (NMsus MIC: 0.5 mg/L), by increasing concentrations of LVX in the medium. Vero cells were inoculated and cultured in medium free of antibiotic until 90% of the cells were infected. The supernatant was removed and infected cells were harvested and centrifuged at 1000× *g* for 15 min. The pellet was resuspended in fresh culture medium, and the cells were disrupted by three freeze−thaw cycles (from −210 to 37 °C). After centrifugation at 1000× *g* for 15 min, the supernatant with the liberated bacteria from the infected cells was used to infect fresh Vero cell monolayers. The inoculum was removed after 1 h of incubation at 35 °C in a 5% CO_2 atmosphere, the cells were washed with culture medium in order to eliminate the unphagocytosed bacteria, and fresh medium containing 0.2 mg of LVX per liter, was added. The monolayer was incubated at 35 °C in a 5% CO_2 atmosphere. The supernatant was removed every three days, and fresh medium containing 0.2 mg of LVX per liter was added. The same procedure was followed with constantly increasing concentrations of the antibiotic until the MIC reached 32 mg/L, as determined by the shell-vial assay at which point bacteria were collected for inoculation titration test and MIC determination.[24] Parallel cultures of the same strain (NMsus) were kept at all times undergoing the same experimental procedures with absence of the antibiotic so as to serve as the susceptible strain.

Antibiotic Preparation. The antibiotics used in this study were Levofloxacin (LVX) (Tavanic i. v. 500, Aventis Pharma Deutschland GmbH) and Ciprofloxacin (CIP) (Topistin i.v. ELPEN Pharmaceutical Co, Inc. Attica Greece). LVX was directly diluted into freshly prepared culture medium to reach the appropriate concentration. Culture media containing the antibiotic were sterilized by filtration (pore size: 0.22 μm) and stored at 4 °C. Both antibiotics were shown, by shell vial assays (data not included), to be nontoxic to Vero cells for concentrations up to 128 mg/L.

***C. burnetii* Inoculation Titration Test and MIC Determination.** *C. burnetii* inoculation titration test and Minimum Inhibitory Concentration determination were performed as described,[25,26] with minor modifications. In brief, cell cultures that reached infection level of around 90% were trypsinized and resuspended in culture medium. Following strong homogenization including a number of passages through a fine syringe needle, the infected cell suspension containing free bacteria was centrifuged at 4 °C for 15 min at 500 g. The supernatant, mainly containing free bacteria (as determined by Gimenez staining) was stored at −80 °C in 0.5 mL fractions. In order to

establish the number of infecting units per ml of the stored fractions, dilutions (10^{-1} to 10^{-12}) of the fractions were tested by infecting confluent monolayers of Vero cells in centrifugation shell vials. Following incubation at 35 °C for six days, infected cells were shown by direct immunofluorescence using antiserum (Q fever-Positive control, Panbio Inc.) to *C. burnetii.* The number of infective *C. burnetii* per milliliter of inoculum was calculated from the last dilution that resulted in at least one infected cell. The dilution that would result in 30−50% of the cells becoming infected after inoculation into shell vials was identified to be 10^{-7}. The estimated infecting dose of this dilution was 100 bacteria per shell vial. This dilution was later used for the MIC determination. A series of duplicate shell vials with confluent Vero cells was inoculated with 0.4 mL of the previously determined dilution of the infected cell suspension that would result in 30−50% of the cells becoming infected. Following centrifugation at 30 °C for 1 h at 700× *g*, the supernatant was discarded and 0.1 mL of the antibiotic in concentrations ranging from 0.125−128 mg/L and 0.9 mL of the cell culture medium were added to the shell vials. After six days of incubation at 35 °C in a CO_2 incubator, the MIC of LVX was determined by direct immunofluorescence as the concentration of the antibiotic where there was no intracellular bacteria observed following the six days incubation period. The MIC determination method was also performed for CIP using the NMres strain. Even though during MIC determination tests bacteria were initially challenged by an antibiotic concentration well beyond the MIC mentioned in the literature for *C. burnetii* NM, (Initial Antibiotic Concentration = 0.125 mg/L MIC = 0.5 mg/L), negative controls (no antibiotic present) were always included.

Bacteria and Protein Isolation. Upon reaching the desired level of antibiotic resistance, fluoroquinolone resistant (NMres) and susceptible (NMsus) *C. burnetii* strains were mass cultured in parallel and under identical conditions for 10 days (with the exception of the presence of levofloxacin in NMres) and finally isolated from their host cells using renographin (Ultravist 370; 0.769 g/mL iopromide; Schering) density gradient ultracentrifugation as described elsewhere.[27,28] Renographin clean bacteria resuspended in K36 buffer (16.5 mM KH_2PO_4, 33.5 mM K_2HPO_4, 100 mM KCl, 15.5 mM NaCl) containing a protease inhibitor cocktail (Sigma-Aldrich) underwent five 5-min freeze−thaw cycles (from −210 to 37 °C) and the protein concentration was determined by the Bradford assay. All manipulations involving viable bacteria were performed in a Biosafety Level III laboratory.

Isolation, Identification and Quantification of Differentially Labeled Methionine-Containing Peptides by Combined Fractional Diagonal Chromatography (COFRADIC). The methionine-COFRADIC procedure was performed on both NMres and NMsus whole lysates as described elsewhere.[29] In brief, proteomes were here digested with endoproteinase Lys-C and the peptides were labeled by N-propionylation. The NMsus proteome digest was labeled with $^{12}C_3$-propionyl whereas the NMres proteome digest with $^{13}C_3$-propionyl. Each peptide thus had a label on its N-terminal alpha-amino group and on its C-terminal lysine epsilon-amino group, evoking a difference of 6 Da between light and heavy peptides. Samples were mixed and analyzed by LC−MS/MS on an Ultimate 3000 HPLC system (Dionex, Amsterdam, The Netherlands) in-line connected to a LTQ Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany). Instrument settings for LC−MS/MS analysis and generation of MS/MS peak lists were as described.[30] MS/MS peak lists were searched with Mascot using the Mascot Daemon interface (version 2.2.0, Matrix Science). The Mascot search parameters were as follows. Searches were performed in a *Coxiella burnetii* database downloaded from Uniprot on June 17, 2009 (containing 1,815 protein entries). Lys-C/P was set as the used protease with one missed cleavage allowed, and the mass tolerance on the precursor ion was set to ±10 ppm and on fragment ions to ±0.5 Da. *S*-Carbamidomethylation of cysteine and oxidation of methionine (to its sulfoxide) were set as fixed modifications. In addition, Mascot's C13 setting was set to 1. The light and heavy labels were defined in Mascot's quantitation method. Peptide quantifications were carried out using the Mascot Distiller Quantitation Toolbox (version 2.2.1). The quantification method details were as follows: constrain search: yes, protein ratio type: average, report detail: yes, minimum peptides: 1, protocol: precursor, allow mass time match: yes, allow elution shift: no, all charge states: yes. Ratios for identified proteins were calculated by comparing the XIC peak areas of all matched light peptides with those of the heavy peptides, and the results were verified by visual inspection of MS spectra with the in-house developed Rover tool.[31] All identified MS/MS spectra are publicly available in the PRIDE database (http:www.ebi.ac.uk/pride) under the experiment number 13081 (note that currently only referees can examine these data using the login "review38464" and password).

Results and Discussion

In vitro Production of Levofloxacin-Resistant *C. burnetii*. The increased use of fluoroquinolones as a therapeutic agent for a wide spectrum of infections has led to increasing resistance to these antimicrobials. Resistance to fluoroquinolones typically arises as a result of alterations in the target enzymes (DNA gyrase and topoisomerase IV) and of changes in drug entry and efflux. For acute Q fever, bacteriostatic antibiotics are useful in helping a patient recover, but to cure a patient with chronic Q fever, a physician should consider the use of a bactericidal antibiotic regimen.[32] Various antibiotic regimens, including the quinolones, have been proposed for the treatment of acute Q fever, but none of these antibiotics is bactericidal *in vitro* which may, in part, explain failures in treating chronic Q fever infections.[32,33]

Levofloxacin (LVX), the L-isomer of ofloxacin, is approximately twice as active as ofloxacin against most Gram-positive and Gram-negative bacteria and has improved intracellular pharmacokinetic and pharmacodynamic properties. It is a reliable alternative to tetracycline therapy not only for acute Q fever but for Mediterranean spotted fever and scrub typhus as well.[24]

C. burnetii reference strain (Nine Mile phase II) presented MIC of 0.5 mg/L for LVX. *C. burnetii* NM was converted to LVX-resistant strain in *in vitro* conditions (NMres). First-step resistant bacteria (MIC 1 mg/L) were obtained after circa five weeks of passage with 0.5 mg/L LVX. Final step resistant bacteria (MIC 32 mg/L) were obtained after 26 additional weeks of passage with increasing concentrations of LVX in the culture medium (Table 1). Upon achievement of the desired MIC, mass cultivation (twenty 225 cm^2 flasks) of NMres with culture medium containing the analogous concentration of the antibiotic present was started, in parallel with NMsus without the presence of the antibiotic. Vero isolated-bacteria provided the minimum amount of protein required (8 mg of protein) for the proteomic analysis.

Table 1. Minimum Inhibitory Concentrations of Levofloxacine During the Time Period of the *in vitro* Production of Levofloxacin-Resistant *C. burnetii* as Determined by the Shell Vial Assay

week no.	[LVX] in culture medium	MIC (μg mL^{-1}) NMres	MIC (μg mL^{-1}) NMsus
5	0.5	1	0.5
8	1	2	0.5
12	2	4	0.5
17	4	8	0.5
21	8	16	0.5
31	16	32	0.5

The MIC of NMres (MIC 32 mg/L) was tested against CIP as well to verify cross resistance among quinolones. The difference between the MICs of two quinolones was a one step lower concentration for CIP (16 mg/L) indicating the existence of cross-resistance among ciprofloxacin and levofloxacin in *C. burnetii.*

Protein Identification. Other than an established association of a high level of resistance to quinolones, nucleotide mutations in QRDRs[20−22] and an observed decreased intracellular accumulation in quinolone-resistant strains,[7] little is known on the protein level about the means of resistance to fluoroquinolones in *C. burnetii.* Out of the 9160 MS/MS-spectra identified, 7166 contained methionine, providing 1419 unique peptides and 660 unique proteins. However, when using highly accurate peptide masses, Mascot identification threshold tends to be too low and thus if the entire list of identifications was considered, too many false positives were expected to get included. Therefore, peptides that were more likely to be false positives were left out. These included peptides shorter than 8 amino acids, peptides with identity score values that were not greater than ten units compared to the threshold, and finally peptides with a falsely calculated ratio as judged by the quantification module. This filtration resulted in the identification of 381 unique proteins common to both strains (Supplementary Table 1, Supporting Information). The set of 381 proteins with a validated N-terminal peptide were assigned to 20 functional categories (Supplementary Table 1, Supporting Information) based on the criteria of Samoilis et al.[27] Although the applied method has a bias toward abundantly expressed proteins, the high density of the data set ensures that a large number of low abundance proteins are included. Furthermore, the protein set covers a wide range of cellular functions, which also suggests an unbiased estimation of the *in vivo* expressed proteins. Two dimensional gel electrophoresis techniques have previously been used for proteomic studies of *C. burnetii.*[27,34−36] This strategy however has limitations because low abundance and/or very basic proteins are difficult to detect. Using the COFRADIC strategy described herein, the mean p*I* value of the proteins not previously detected in *C. burnetii* was 8.4.

Differently Expressed Proteins and Their Cellular Function. Statistical analysis was done on the log2 of the peptide ratios. The log2 mean of the ratios was 0.064, meaning that the peptides are centered around 1:1 ratio. The standard deviation was 0.718, thus 95% of the peptides have a log2 ratio between −1.343 and 1.471 or a normal ratio between 0.394 and 2.773. The peptides and proteins out of this interval are considered to be significantly up or down-regulated, with high ratios indicating proteins that are more abundant in the NMsus strain, whereas proteins with low ratios are more abundant in the NMres strain. Out of the 381 proteins, 13 were up-regulated in NMsus and 2 were up-regulated in NMres (Figure 1 and Table 2).

Thirteen proteins were up-regulated in NMsus. The largest group includes five proteins (Q45881, B5QSC0, Q83DY0, B5QSC3 and Q83DK8) with unknown function, the next group includes two proteins (Q83CH9 and Q83CH7) associated with pathogenesis while one protein has been identified to be involved in DNA metabolism (Q83F84), purine biosynthesis (Q83DB4), adaptation (Q83D86), biosynthesis of cofactors (Q83AR8), cell division (Q81ZL2) and protein fate (Q83BT6). In the case of the NMres only two proteins were upregulated one involved in the detoxification process (Q83DB9) and in fatty acid and phospholipid metabolism (Q820W7). More than 50% of the differently expressed proteins have pI value higher than nine underlining the advantage of COFRADIC to study basic proteomes.

In the case of NMsus, two of the five proteins with unknown function have extremely basic p*I*s. The Q45881 with a p*I* of 12.54 has been annotated as a Histone-like protein and the second the small cell variant protein A (B5QSC0, p*I* 12.13). The Histone-like protein (Q45881) is a developmental stage-specific histone H1 homologue of *Coxiella burnetii* and it has been

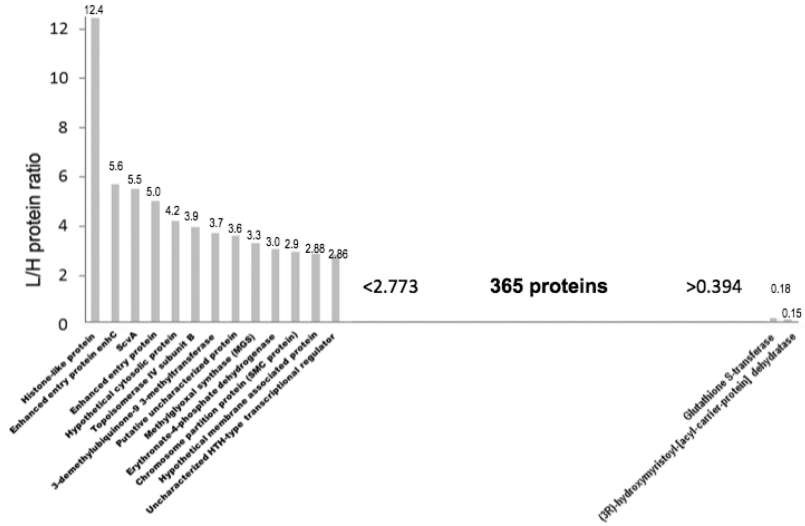

Figure 1. Identified proteins, which are up regulated.

Table 2. Identified Proteins that were Significantly Up-Regulated in Either of the Two Strains

gene locus	accession no.	description	L/H ratio[a]	Nm of spectra/ peptides	overexpressed	MW kDa	pI	GRAVY score[b]	cellular localization	cellular role category
CBU_0456	Q45881	Histone-like protein	12.44	14/4	NMsus	13.1	12.59	−1.646	Unknown	Unknown
CBU_1136	Q83CH9	Enhanced entry protein enhC	5.69	8/4	NMsus	117.7	9.34	−0.471	Extracellular	Pathogenesis
CBU_1267.1	B5QSC0	ScvA	5.50	520/3	NMsus	3.6	12.13	−2.583	Unknown	Unknown
CBU_1138	Q83CH7	Enhanced entry protein	5.04	3/1	NMsus	27.7	9.07	−0.298	Noncytoplasmic	Pathogenesis
CBU_0560	Q83DY0	Hypothetical cytosolic protein	4.20	5/2	NMsus	46.4	6.57	−0.14	Unknown	Unknown
CBU_0064	Q83F84	Topoisomerase IV subunit B	3.97	12/5	NMsus	71.5	6.87	−0.364	Cytoplasmic	DNA metabolism: DNA replication, recombination, and repair
CBU_0350	Q83DB4	Adenylosuccinate lyase	3.71	6/2	NMsus	52.5	7.22	−0.284	Unknown	Purine ribonucleotide biosynthesis
CBU_1280.1	B5QSC3	Putative uncharacterized protein	3.62	4/1	NMsus	8.8	9.8	−0.339	Unknown	Unknown
CBU_0853	Q83D86	Methylglyoxal synthase	3.33	5/2	NMsus	16.2	6.04	−0.215	Cytoplasmic	Adaptations to atypical conditions
CBU_1812	Q83AR8	Erythronate-4-phosphate dehydrogenase	3.07	5/1	NMsus	40.9	6.5	−0.059	Cytoplasmic	Biosynthesis of cofactors, prosthetic groups, and carriers: Pyridoxine
CBU_0540	Q81ZL2	Chromosome partition protein	2.96	16/4	NMsus	133.5	5.27	−0.691	Cytoplasmic	Cell division
CBU_0718	Q83DK8	Hypothetical membrane associated protein	2.88	115/6	NMsus	10.4	10.06	−0.401	Unknown	Unknown
CBU_1416	Q83BT6	Uncharacterized HTH-type transcriptional regulator	2.86	3/2	NMsus	24.2	6.85	−0.309	Unknown	Protein fate: Degradation of proteins, peptides, and glycopeptides
CBU_0819	Q83DB9	Glutathione *S*-transferase	0.18	2/2	NMres	25.4	8.89	−0.13	Unknown	Detoxification
CBU_0614	Q820W7	3-hydroxymyristoyl-dehydratase	0.15	16/5	NMres	16.3	9.35	0.097	Cytoplasmic	Fatty acid and phospholipid metabolism: Biosynthesis

[a] Ratio between light labeled ($^{12}C_3$-propionyl -NMsus) and heavy labeled ($^{13}C_3$-propionyl-NMres) bacterial strain. [b] GRAVY score is the average hydropathy score for all the amino acids in the protein. Integral membrane proteins typically have higher GRAVY scores than do globular proteins.

suggested that is more abundant in the metabolically dormant small cell variant (SCV).[37] In addition, the overexpression of the ScvA (B5QSC0), a small cell variant-specific protein in the NMsus allow us to speculate that the SCV are predominantly present in the NMsus compared to the NMres. Biological confirmation by determining the LCV to SCV ratio using electron microscopy could provide verification whether levofloxacin-resistant *C. burnetii* strain indicates a smaller proportion of small cell variants population when compared to the antibiotic susceptible strain thus, holding a different, and metabolically more active, intracellular profile for parasitism.

The identification of one of the targets of fluoroquinolones, topoisomerase IV subunit B (Q83F84), overexpressed in NMsus is of particular interest. Although mutations at the binding sites of the particular enzyme (QRDR) as a fluoroquinolone resistance mechanism in pathogens have been described,[38] this is not the case with *C. burnetii* where recent work has shown that nucleotide mutations within QRDRs of *C. burnetii* are observed in all the subunits of gyrase and topoisomerase apart from the subunit B of topoisomerase IV.[22] Thus, under-expression of this particular enzyme in the NMres strain indicates a reduction in DNA replication due to the fluoroquinolone action.

As an obligatory intracellular pathogen, the invasion of *C. burnetii* of its host cell is considered a significant virulence determinant. The enhanced entry proteins (Q83CH9 and Q83CH7) found to be more abundant in NMsus compared to NMres could ultimately prove of importance in the invasion ability of the pathogen. However there is no firm evidence thus far that these proteins aid the bacterium toward its invasion in eukaryotic cells. The overexpression of the enhanced entry proteins in NMsus further point to a potentially reduced ability of the fluoroquinolone resistant strain to invade host cells. Several studies on Salmonella strains have indicated a possible link between fluoroquinolone resistance and decreased cell invasion ability.[39−41] Several hypotheses were put forward to explain this phenomenon, which partly explains the observed low prevalence of fluoroquinolone-resistant Salmonella clinical isolates.[39] The most important one is that exposure to quinolones leading to a high-level of resistance may alter growth rate, and this may be the connecting factor triggering coordinated repression of genes implicated in the invasion phenotype since the optimal environmental conditions for the expression of these genes is lost.[39] Additional experiments need to be performed in order to assess whether the enhanced entry proteins of *C. burnetii* aid the bacterium toward its entry into the eukaryotic cell and evaluate the connection between fluoroquinolone resistance and decreased cell invasion ability.

One of the two proteins identified to be in higher abundance in NMres, glutathione *S*-transferase (GST, Q83DB9) is of particular interest. Bacterial GSTs are part of a superfamily of enzymes that play a key role in cellular detoxification. GSTs are widely distributed in prokaryotes and are grouped into several classes. Bacterial GSTs are implicated in a variety of processes such as the biodegradation of xenobiotics, protection against chemical and oxidative stresses and antimicrobial drug resistance.[42] Several studies on the interaction of GSTs with different classes of antibiotics have been performed and a possible role for the enzyme in antibiotic-resistance has been proposed.[43,44] The higher abundance of GST in the quinolone-resistant strain *C. burnetii* indicates a possible participation of this protein in a bacterial antibiotic resistance molecular mechanism.

Fatty acids are one of the most important building blocks of cells. In bacterial cells, fatty acids occur mainly in the cell membranes as the acyl constituents of phospholipids.[45] The second protein that was found overexpressed in the quinolone-resistant strain, 3-hydroxymyristoyl-dehydratase, is encoded by fabZ and is involved in fatty acid biosynthesis. FabZ, is the first dehydratase which participates in the cycles of elongation leading to unsaturated fatty acid biosyntheses.[46] Moreover, the FabZ substrate, 3-hydroxymyristoyl acyl carrier protein, is situated at a biosynthetic branch point which can also lead to lipid A biosynthesis.[47] Lipid A is the hydrophobic anchor of lipopolysaccharide (LPS). It forms the major lipid component of the outer monolayer of the outer membrane of Gram-negative bacteria and is required for bacterial growth and virulence, and inhibition of its biosynthesis is lethal to bacteria.[48] The observed overexpression of hydroxymyristoyl-dehydratase in the quinolone resistant strain, which is thus of great importance for the biochemical characteristics of *C. burnetii's* membrane, indicates probable alterations of the permeability of the bacterial membrane by the antibiotic—a known bacterial fluoroquinolone-resistance mechanism.

Conclusion

Gram-negative bacteria have been reported to develop resistance to quinolones.[16] This resistance appears to be the result of one of three mechanisms: alterations in the quinolone enzymatic targets (DNA gyrase), decreased outer membrane permeability, or the development of efflux mechanisms. This study presents for the first time, the proteome of an *in vitro* developed fluoroquinolone- resistant *C. burnetii* and its quantitative comparison with that of the respective susceptible strain. The results of this analysis support the participation of detoxification processes indicated by the alteration of the amount of unsaturated lipids in the membranes as a probable mechanism of quinolone resistance. Additionally, the decrease of the expression of developmental stage-specific proteins in the NMres indicates for the first time possible metabolic changes of the resistant strain compared to the NMsus.

Abbreviations: CIP, ciprofloxacine; COFRADIC, combined fractional diagonal chromatography; LCV, large cell variant; LVX, levofloxacin; NM, *C. burnetii* nine mile; NMres, *C. burnetii* nine mile resistant to levofloxacin; NMsus, *C. burnetii* nine mile susceptible to levofloxacin; MIC, minimum inhibitory concentration; SCV, small cell variant; QRDR, quinolone resistance determining region.

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Supporting Information Available: Supplementary Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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