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Proteome analysis of *Mycoplasma fermentans* cultured under aerobic and anaerobic conditions

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Abstract

Background and aims: Mycoplasmas are ubiquitous pathogens found not only in humans but also in animals, plants, insects and soil. Though they usually grow better in an aerobic environment, mycoplasmas are also facultative anaerobic microorganisms. Following infection, the transition of a microorganism from a normal environment into an anaerobic one (e.g. dead or dying tissue) may result in production of a higher number of bacterial toxins. The resolution of the bacterial proteome during the aerobic/anaerobic switch could thus allow the identification of potential pathogenic determinants and pathways.

Methods: We used two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption/ionization time-of-flight/tandem mass spectroscopy (MALDI-TOF MS/MS) and subsequent mass spectrometric analysis to characterize the liposoluble and hydrosoluble protein fractions of a strain of *Mycoplasma fermentans* isolated in our lab (MFI), that was cultured under either aerobic or anaerobic conditions.

Results: We identified the 27 most abundant proteins in the liposoluble fraction and the 30 most abundant proteins in the hydrosoluble fraction and determined their modulation under aerobic and anaerobic growth. By using Protein ANalysis TroughH Evolutionary Relationships (PANTHER) and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) software analysis tools, we were able to identify, define and organize the function of each protein, as well as to determine the specific interactome.

Conclusions: Our work provides the first proteome reference map of *Mycoplasma fermentans* obtained under aerobic and anaerobic growing conditions. These data may help to better understand the mechanisms of pathogenicity of this microorganism and define new diagnostic targets.

Keywords: Mycoplasma, Proteomic analysis, Aerobic, Anaerobic, 2D-electrophoresis

Background

Mycoplasmas are prokaryotic organisms of the class Mollicutes (that literally means “soft skins”) [1] which comprises over 200 species, widely spread in nature as obligate parasites of humans, mammals, reptiles, fish, arthropods, and plants [2]. Their cellular membrane contains phospholipids, glycolipids, sterols and various proteins while lacking a rigid cell wall [2]. These organisms replicate by binary fission and have limited biosynthetic capabilities, depending mostly on the environment (host or growth medium) for the supply of most nutrients. Most of them exist in fact as parasites with strict host and tissue specificities.

Despite their relatively small physical (150 to 350 nm) and genomic size (600–2200 kb) [3–6], mycoplasmas are considered pathogens and cofactors in diverse diseases [7]. Also, mycoplasmas are known to elicit strong host immune responses due to the presence on their membrane of lipoproteins able to trigger a Toll-like receptor-mediated response [8–12]. The variation in mycoplasma lipoproteins attributed

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to genome rearrangements and genetic insertions have been demonstrated to lead to phenotypic plasticity that allows also for the evasion of the host's defense system and pathogenesis [13–15]. Some strains of mycoplasma can invade intracellularly [16–18], including *Mycoplasma fermentans* [19], enhancing their ability to evade the immune system [20]. Given their presence on mostly mucosal surfaces (respiratory and urogenital tracts, eyes, alimentary canal, mammary glands and joints [21–23]), mycoplasmas have been associated to respiratory [24, 25], urogenital [26–28] and rheumatoid diseases [29–31].

In general, mycoplasmas are facultative anaerobes, except for *Mycoplasma pneumoniae*, which is a strict aerobe [21, 32]. The presence of the anaerobic form of these microorganisms is usually explained by the availability of oxygen and by the colonization of microhabitats protected from air. In fact, mycoplasmas are able to invade the deep tissues as a result of mucosal surface disruption, local trauma, surgery, tissue necrosis and impaired clearance of a sterile site. Their ability to grow in anaerobic environments may lead to localized infections [33, 34]. To this regard, in a number of cases mycoplasmas are considered causative agents for localized infections, and the difficulty in their isolation likely renders these associations underestimated [35–39].

Data regarding the constitutive proteome in pathogens under relevant conditions can definitely provide valuable information on their metabolic regulation and pathogenesis. In this study, we performed the proteome analysis of a strain of *Mycoplasma fermentans* MFI, isolated in our laboratory from samples obtained from an HIV-seropositive patient [40]. This strain of *Mycoplasma fermentans* has a nucleotide sequence very similar to another strain isolated in our lab (MFII) and to the prototypes *Mycoplasma fermentans* PG18 and M64 [3, 41, 42]. We chose to study the strain MFI because its DnaK showed broad anticancer properties both in vivo and in vitro [40]. To this regard we demonstrated that this mycoplasma promoted lymphomagenesis in an in vivo mouse model and its bacterial chaperone protein, DnaK, reduced the activity of PARP1 and p53, involved in DNA damage control/repair and cell-cycle/apoptosis, respectively [40]. Protein expression profiles of this microorganism cultured in aerobic and anaerobic conditions were determined using two-dimensional gel electrophoresis (2-DE) and proteins were further identified using MALDI-TOF/MS-MS.

The present work provides unique information regarding the proteome of *Mycoplasma fermentans* MFI isolate grown in two different conditions. Our data may help to identify potential candidates for functional studies and better elucidate pathways related to pathogenesis, as well as possible virulence-associated diagnostic and therapeutic targets.

Methods

Bacterial strains and culture conditions

In this study we analyzed the strain of *Mycoplasma fermentans* MFI isolated in our laboratory from samples of an HIV-infected patient [40]. The whole genome shotgun sequence of strain MFI was obtained with the Roche 454 FLX pyrosequencing technology at ~75x and ~45x coverage, respectively. Raw reads were assembled into contigs using Newbler v1.1.03.24 then annotated using the Institute for Genome Sciences automated Annotation Engine pipeline (http://ae.igs.umaryland.edu/). The annotated whole genome sequence has been deposited at the NCBI Whole Genome Shotgun (WGS) repository (MF-I1 - ATFG00000000).

Laboratory isolates of MFI were grown both in anaerobic and aerobic conditions. The anaerobic growth was performed in PPLO medium (BD) supplemented with 17% heat inactivated fetal bovine serum (ThermoFisher Scientific), tryptone (BD) 1%, glucose (Sigma-Aldrich) 0.5%, yeast extract solution 3.5% and yeastolate 1%, at 37 °C (both from BD). Anaerobic conditions were achieved by growing the cultures inside anaerobic jars containing GasPaks (BD). The aerobic growth was performed in 243 medium containing heart infusion broth (BD Biosciences) supplemented with 20% heat inactivated horse serum and 10% yeast extract solution (ThermoFisher Scientific), at 37 °C. Mycoplasma cultures were harvested in late log phase, collected by centrifugation (20 min at 10,000 g at 4 °C), and washed three times with PBS. At least three mycoplasma pellets were obtained from each bacterial culture replicate, and used for proteomic analysis.

Protein extraction and triton X-114 fractionation

For total protein extracts, bacterial pellets were resuspended in 1% hot SDS, incubated for 3 min at 95 °C, chilled and diluted with lysis buffer (10 mM Tris HCl pH 7.4, 0.15 M NaCl, 1 mM EDTA, PBS, 1% V/v Triton X-114 and protease inhibitors). Lysates were sonicated for 5 min on ice-water mix and insoluble material was removed by centrifugation (30 min at 10,000 g at 4 °C). Hydrophilic and hydrophobic protein fractions were obtained by Triton X-114 fractionation [43, 44].

Delipidation and protein precipitation by chloroform/methanol/water

The method developed from Wessel [44, 45] was used for delipidation and protein precipitation. Delipidated protein pellets were air-dried and resuspended in

ReadyPrep protein extraction kit from Biorad, following the instructions from the manufacture. Protein samples were quantified using RC DC protein assay kit (Biorad).

2D-Dige proteomics profile of mycoplasmas

We used 2D-Dige analysis followed by MALDI-TOF/MS-MS protein identification to analyze the proteomic profile of MFI growth in aerobic versus anaerobic conditions. Proteomics profile was performed by Applied Biomics, following a protocol previously published [46]. Briefly, delipidated hydrophilic and hydrophobic protein fractions from MFI growth either in aerobic condition or in anaerobic condition were labeled with Cy3 or Cy5 dyes, and a 1:1 mix was also labeled with Cy2 which was included with the test samples as an internal labeling control. Samples were then analyzed using an Amersham Biosciences 2D-gel system (Amersham Biosciences). DIGE images were detected with a Typhoon TRIO scanner, analyzed by ImageQuant software version 5.0 (GE Healthcare) and processed with DeCyder software version 6.5 (GE Healthcare) for image analysis. Student's t test analysis was used for statistical analysis, and significant change of protein abundance was defined as at least 1.5-fold difference ($P < 0.05$). NCBI and MFI library were used as sequence databases. The detailed protocol of the proteomic analysis procedure is available elsewhere [46].

Functional and biological characterization of the proteins

MS data were subjected to gene ontology analysis with Blast2GO [47] and used to generate tables containing information regarding protein identity, molecular weight, pI (isoelectric point), peptides count, identity score and accession number. Identified proteins were then classified based on cellular components, protein class, biological processes and molecular functions using the PANTHER bioinformatics tool (v. 9.0; http://www.pantherdb.org) [48, 49]. Finally the biological associations among the proteins from the aerobic and anaerobic growth conditions were investigated using the online STRING software (v. 10; https://string-db.org) [50]. A medium confidence score of 0.4 was used as the cutoff criterion.

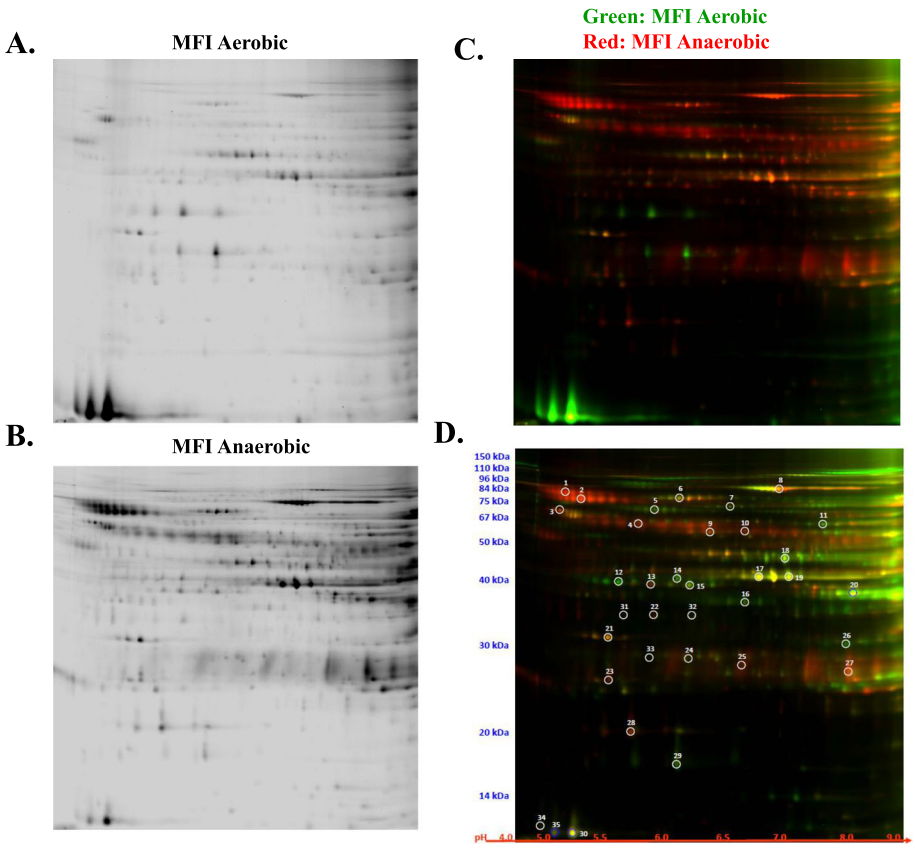

Fig. 1 **a**: 2D-Dige Triton X-114 liposoluble fractions of *Mycoplasma fermentans* MFI, grown in aerobic and **b**: anaerobic conditions. **c**: Superimposition of the signals generated by 2D-Dige Triton X-114 liposoluble fractions of MFI. Growing conditions are indicated in figure with red, anaerobic, and green, aerobic. **d**: 2D-Dige gel map of MFI liposoluble proteins showing the protein identification number obtained by mass spectrometry

Results

2D-electrophoresis protein comparison and quantification from *Mycoplasma fermentans* cultured in aerobic and anaerobic conditions

We used a 2D-Dige electrophoresis to characterize quantitatively and qualitatively the protein profile of *Mycoplasma fermentans* grown in aerobic and anaerobic conditions. A specific procedure was employed to separate proteins soluble in lipids (liposoluble fraction) or soluble in water (hydrosoluble fraction). The liposoluble (Fig. 1a-c) and hydrosoluble (Fig. 2a-c) fractions from aerobic and anaerobic culture conditions were compared by superimposition of the signals to the corresponding fraction. By using mass spectrometry, we identified the 35 (27 of which were uniquely identified) most abundant proteins differentially expressed in the liposoluble fraction (Table 1) and the 38 (30 of which were uniquely identified) most abundant proteins in the hydrosoluble fraction (Table 2). A few proteins were identified in both fractions, most likely because of a cross-contamination between the two phases due to their abundance. We calculated the ratio of the single protein expression in anaerobic versus aerobic culture conditions (see Material and Methods). These values are shown in Table 3a (for the liposoluble fraction) and Table 3b (for the hydrosoluble fraction). The differential expression of the isolated proteins in anaerobic and aerobic conditions is illustrated in Fig. 3a (for the liposoluble fraction) and Fig. 3b (for the hydrosoluble fraction). The higher ratio of the enzyme arginine deaminase (arcA) in the anaerobic fraction (Fig. 3a) indicated that our experimental procedures were correct, since this enzyme is a repressor of various aerobic enzymes [51].

A remarkable difference between the two isolated fractions consisted in the high protein ratio of anaerobic versus aerobic observed in the liposoluble fraction versus the hydrosoluble (Fig. 3). In particular, we observed minor differences in the hydrosoluble protein ratio between the anaerobic and aerobic grown mycoplasma with an average ratio ranging from − 2.58 to 3.53. In contrast, we saw large differences in the expression of liposoluble proteins in anaerobic versus aerobic, with an average ratio ranging from − 21.61 to 8.05. The homogenous expression in aerobic and anaerobic mycoplasma hydrosoluble proteins suggests

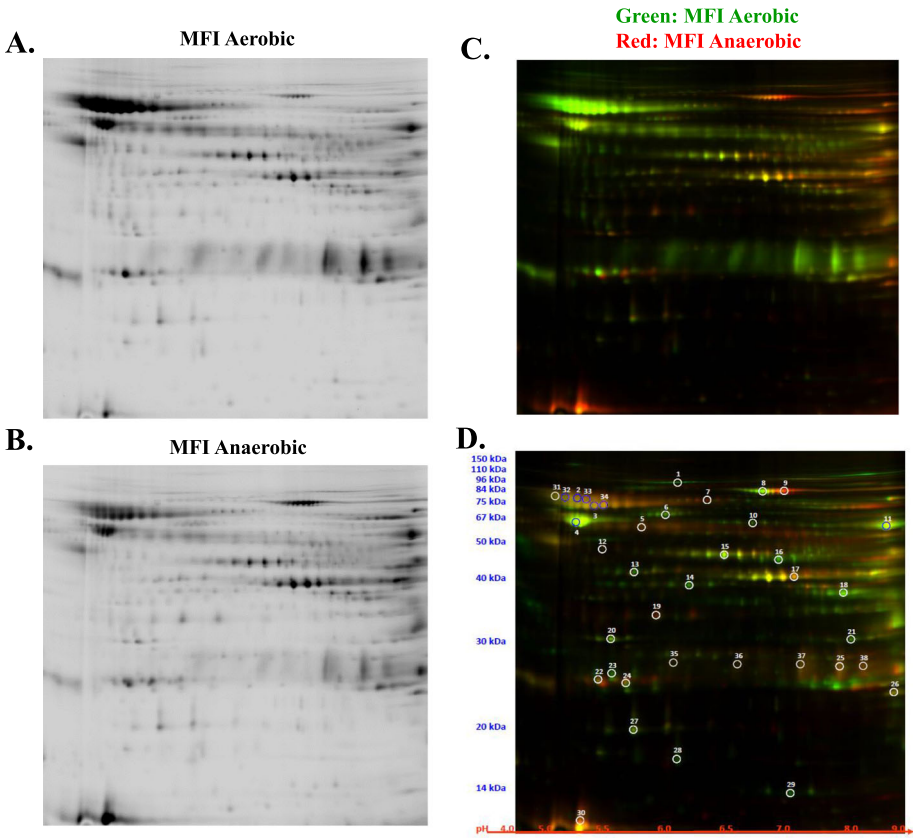

Fig. 2 a: 2D-Dige Triton X-114 hydrosoluble fractions of *Mycoplasma fermentans* MFI, grown in aerobic and **b**: anaerobic conditions. **c**: Superimposition of the signals generated by 2D-Dige Triton X-114 hydrosoluble fractions of MFI. Growing conditions are indicated in figure with red, anaerobic, and green, aerobic. **d**: 2D-Dige gel map of MFI hydrosoluble proteins showing the protein identification number obtained by mass spectrometry

Table 1 List of proteins identified in the liposoluble fractionof *Mycoplasma fermentans* MFI proteome obtained from 2D page Triton X-114

spot	protein ID	Mw (Da)	pI	count	score	Acc. No.	New Acc. No.	gene
1	conserved hypothetical protein / lipoprotein	10,984	9.9	4	< 95	gi|308,190,339|ref.|YP_003923270.1|	WP_013355024.1	lspA
2	sucrase-isomaltase	33,221.2	7.0	6	< 95	gi|308,190,125|ref.|YP_003923056.1|	WP_013354811.1	malL
3	aspartate-ammonia ligase	39,505.8	8.9	6	< 95	gi|308,189,759|ref.|YP_003922690.1|	WP_013354447.1	asnA
4	excinuclease ABC subunit C	57,716.9	8.7	9	< 95	gi|308,190,195|ref.|YP_003923126.1|	WP_013354881.1	uvrC
5	unidentified protein (39aa)	4542.5	10.2	5	< 95			[a]nd
6	elongation factor G	77,217.8	5.7	31	100	gi|148,377,855|ref.|YP_001256731.1	WP_011949750.1	fusA
7	DNA topoisomerase (ATP-hydrolyzing) subunit B	42,632.7	5.4	14	100	gi|308,190,314|ref.|YP_003923245.1|	WP_013354999.1	parE
8	phosphoketolase family protein	89,889.5	6.4	32	100	gi|308,189,603|ref.|YP_003922534.1|	WP_013354291.1	xfp
9	ATP-binding cassette domain-containing protein	47,304.9	6.0	16	100	gi|308,190,277|ref.|YP_003923208.1|	WP_013354963.1	potA
10	histidine-tRNA ligase	8705.7	10.0	3	< 95	gi|308,190,116|ref.|YP_003923047.1|	WP_013354802.1	hisS
11	lysine-tRNA ligase	34,794.3	9.3	12	100	gi|308,189,610|ref.|YP_003922541.1|	WP_013354298.1	lysS
12–13	DNA-directed RNA polymerase alpha chain	38,023.8	5.6	23	100	gi|308,190,154|ref.|YP_003923085.1|	WP_013354840.1	rpoA
14–17–19-33	elongation factor Tu (EF-Tu)	43,620.5	6.3	78	100	gi|308,189,768|ref.|YP_003922699.1|	WP_013354456.1	tuf
15	pyruvate dehydrogenase (acetyl-transferring) E1 component subunit alpha	31,168.1	9.5	8	100	gi|308,190,218|ref.|YP_003923149.1|	WP_013354904.1	pdhA
16	bifunctional oligoribonuclease/PAP phosphatase NrnA	20,844.6	6.4	8	100	gi|308,189,648|ref.|YP_003922579.1|	WP_013354336.1	nrnA
18	ornithine carbamoyltransferase	38,356.7	8.0	23	100	gi|308,189,964|ref.|YP_003922895.1|	WP_013354652.1	argF
20	arginine deiminase	31,623.3	5.8	14	100	gi|308,189,965|ref.|YP_003922896.1|	WP_013354653.1	arcA
21	YebC/PmpR family DNA-binding transcriptional regulator	19,896.7	4.5	7	100	gi|308,189,601|ref.|YP_003922532.1|	WP_013354289.1	pmpr
22–31-32	DNA polymerase III subunit gamma/tau	29,077.1	4.6	8	100	gi|308,190,298|ref.|YP_003923229.1|	WP_013354984.1	dnaX
23	elongation factor P	20,893.7	5.2	8	100	gi|148,377,574|ref.|YP_001256450.1|	WP_004024464.1	efp
24	HAD family phosphatase	28,122.2	7.0	15	100	gi|308,189,859|ref.|YP_003922790.1|	WP_013354547.1	had
25	DNA repair protein HhH-GPD	19,566.4	7.7	6	< 95	gi|308,189,903|ref.|YP_003922834.1|	WP_013354591.1	p37
26	5-dehydro-4-deoxy-D-glucuronate isomerase	31,625.1	8.2	17	100	gi|308,190,016|ref.|YP_003922947.1|	WP_013354704.1	kduI
27	hyalorunate lyase	8234.2	6.1	4	< 95	gi|308,190,026|ref.|YP_003922957.1|	WP_013354714.1	hysA
28	transcription elongation factor GreA	6863.6	5.1	5	100	gi|308,189,955|ref.|YP_003922886.1|	WP_013354643.1	greA
29	nucleoside 2-deoxyribosyltransferase	18,332.4	5.8	12	100	gi|319,777,718|ref.|YP_004137369.1|	WP_013355007.1	ndt
30–34-35	50s ribosomal protein L7/L12	12,807.9	5.1	17	100	gi|308,190,181|ref.|YP_003923112.1|	WP_013354867.1	rplL

The score number corresponds to the confidence of the protein ID: scores above 95% are significant; lower scores (< 95%) may be correct, but should require more evidence. The count represents the number of peptides that match the protein

[a]*nd* not determined

Table 2 List of proteins identified in the hydrosoluble fractionof *Mycoplasma fermentans* MFI proteome obtained from 2D page Triton X-114

spot	protein ID	Mw (Da)	pI	count	score	Acc. No.	New Acc. No.	gene
1	ABC transporter ATP-binding protein	41,298.6	7.83	11	100	gi|319,777,349|ref.|YP_004137000.1|	WP_013354741.1	abca
2–3–12-31-32-33-34	choline kinase	40,261.00	8.44	25	< 95	gi|308,189,726|ref.|YP_003922657.1|	WP_013354414.1	licA
4	chaperone protein dnaK	58,875.1	5.43	21	100	gi|308,189,677|ref.|YP_003922608.1|	WP_013354365.1	dnak
5	ATP-dependent DNA helicase PcrA	25,219.4	7.96	6	< 95	gi|308,189,989|ref.|YP_003922920.1|	WP_013354677.1	pcrA
6	translation initiation factor IF-2	66,485.8	6.71	10	< 95	gi|308,189,652|ref.|YP_003922583.1|	WP_013354340.1	infB
7	elongation factor G	77,217.8	5.74	24	100	gi|148,377,855|ref.|YP_001256731.1|	WP_011949750.1	fusA
8–9	phosphoketolase family protein	89,889.5	6.39	57	100	gi|308,189,603|ref.|YP_003922534.1|	WP_013354291.1	xfp
10	phosphoenolpyruvate--protein phosphotransferase	48,282.4	7.67	14	100	gi|308,189,969|ref.|YP_003922900.1|	WP_013354657.1	ptsI
11	peptidylprolyl isomerase (trigger factor)	18,722.4	8.54	8	100	gi|308,189,700|ref.|YP_003922631.1|	WP_013354388.1	tig
13	peptide chain release factor 1	39,782.6	5.40	11	100	gi|308,190,290|ref.|YP_003923221.1|	WP_013354976.1	prfA
14	pyruvate dehydrogenase (acetyl-transferring) E1 component subunit alpha	31,168.1	9.45	13	100	gi|308,190,218|ref.|YP_003923149.1|	WP_013354904.1	pdhA
15	phosphopyruvate hydratase/enolase	17,582.9	4.86	8	100	gi|308,189,766|ref.|YP_003922697.1|	WP_013354454.1	eno
16	arginine deiminase	31,623.3	5.82	14	100	gi|308,189,965|ref.|YP_003922896.1|	WP_013354653.1	arcA
17	elongation factor Tu (EF-Tu)	43,620.5	6.30	25	100	gi|308,189,768|ref.|YP_003922699.1|	WP_013354456.1	tuf
18	ornithine carbamoyltransferase	38,356.7	8.03	23	100	gi|308,189,964|ref.|YP_003922895.1|	WP_013354652.1	argF
19	DNA polymerase III subunit gamma/tau	29,077.1	4.63	3	100	gi|308,190,298|ref.|YP_003923229.1|	WP_013354984.1	dnaX
20	YebC/PmpR family DNA-binding transcriptional regulator	19,896.7	4.45	7	100	gi|308,189,601|ref.|YP_003922532.1|	WP_013354289.1	pmpr
21	class II fructose-1,6-bisphosphate aldolase	30,822	8.35	15	100	gi|308,189,732|ref.|YP_003922663.1|	WP_013354420.1	fba
22	23S rRNA (guanosine(2251)-2′-O)-methyltransferase RlmB	25,771.6	8.89	3	< 95	gi|308,190,283|ref.|YP_003923214.1|	WP_013354969.1	rlmb
23	DNA polymerase III subunit alpha	32,241.8	9.18	9	< 95	gi|308,190,227|ref.|YP_003923158.1|	WP_013354913.1	dnaE
24	ribosome recycling factor	20,417.7	6.20	8	100	gi|308,190,230|ref.|YP_003923161.1|	WP_013354916.1	frr
25	hyalorunate lyase	8234.2	6.13	4	< 95	gi|308,190,026|ref.|YP_003922957.1|	WP_013354714.1	hysA
26	hypoxanthine phosphoribosyltransferase	14,458.7	8.78	9	100	gi|308,189,952|ref.|YP_003922883.1|	WP_013354640.1	hpt
27	transcription elongation factor GreA	6863.6	5.05	5	100	gi|308,189,955|ref.|YP_003922886.1|	WP_013354643.1	greA
28	nucleoside 2-deoxyribosyltransferase	18,332.4	5.78	11	100	gi|319,777,718|ref.|YP_004137369.1|	WP_013355007.1	ndt
29	RpiB/LacA/LacB family sugar-phosphate isomerase	14,164.1	6.58	9	100	gi|308,189,658|ref.|YP_003922589.1|	WP_013354346.1	rpiB
30	50s ribosomal protein L7/L12	12,807.9	5.08	6	100	gi|308,190,181|ref.|YP_003923112.1|	WP_013354867.1	rplL
35	phosphate ABC transporter	38,776.9	8.9	6	< 95	gi|308,190,268|ref.|YP_003923199.1|	WP_013354954.1	pstB
36–38	molecular chaperone DnaJ	24,597.1	9.1	13	< 95	gi|308,189,684|ref.|YP_003922615.1|	WP_013354372.1	dnaJ
37	30S ribosomal protein S2	22,851	9.1	3	< 95	gi|308,189,983|ref.|YP_003922914.1|	WP_013354671.1	rpsB

The score number corresponds to the confidence of the protein ID: scores above 95% are significant; lower scores (< 95%) may be correct, but should require more evidence. The count represents the number of peptides that match the protein

Table 3 Protein ratio of liposoluble (A) and hydrosoluble (B) fraction of *Mycoplasma fermentans*. The ratio MFI anaerobic versus MFI aerobic is shown

A. Liposoluble proteins		
spot#	gene	MFI An/MFI Ae
1	lspA	48.6
2	malL	27.89
3	asnA	4.78
4	uvrC	11.46
5	*nd	1.55
6	fusA	2.31
8	xfp	5.59
9	potA	7.4
10	hisS	11.92
11	lysS	−1.35
12–13	rpoA	from −1.85 to −1.04
14–17–19-33	tuf	from −29.58 to 1.74
15	pdhA	3.22
16	nrnA	1.11
18	argF	3.29
20	arcA	2.17
22–31-32	dnaX	from −22.34 to −21.41
23	efp	1.69
24	had	−11.04
25	p37	5.91
26	kdul	−1.8
27	hysA	5.56
28	greA	2.72
30–34-35	rplL	from −83.41 to −43.26
B. Hydrosoluble proteins		
spot#	gene	MFI An/MFI Ae
1	abca	1.16
2–3–12-31-32-33-34	licA	from −4.38 to −2.85
4	dnak	−1.69
5	pcrA	−1.74
6	infB	−1.55
8–9	xfp	from 3.22 to 15.14
10	ptsI	1.01
11	tig	1.3
13	prfA	−1.29
15	eno	− 1.39
17	tuf	3.52
19	dnaX	3.34
20	pmpr	2.11
21	fba	2.7
22	rlmb	−3.69
23	dnaE	−1.33
24	frr	4.6
25	hysA	−2.64
26	hpt	4.84
28	ndt	−1.89
29	rpiB	2.6
30	rplL	9.82
35	pstB	−3.12
36–38	dnaJ	from −3.11 to −2.29
37	rpsB	−2.7

A cut-off value of ±1.5 was used to distinguish between proteins similarly expressed in aerobic and anaerobic conditions ($-1.5 \leq \text{ratio} \leq +1.5$) from proteins comparatively more expressed (ratio > + 1.5 and < − 1.5). When the same protein was found in both fractions, only the spot with higher ratio, consisting with the most represented protein, was included

that this fraction mostly contains proteins essential for the life of the bacteria, while the substantial changes in the expression of liposoluble proteins in anaerobic versus aerobic, indicates that this fraction includes proteins necessary for the growth of mycoplasma in the presence or the absence of oxygen.

Functional classification of proteins

The identified proteins from anaerobic and aerobic growth were classified using the PANTHER software according to the Gene Ontology (GO) annotations for protein classes and molecular functions [48, 49] (Fig. 4 and Additional file 1: Table S1). *Mycoplasma genitalium* was the only mycoplasma specie annotated in PANTHER, therefore it was used as a reference for these classifications.

When the proteins were classified according to their class (Fig. 4a-b), the dominant class for both culture conditions consisted in the nucleic acid binding proteins (Fig. 4a 46.7% anaerobic and Fig. 4b 40% aerobic, respectively). A striking difference between the two culture conditions was that proteins with isomerase activity were only found in mycoplasma grown in anaerobic conditions (12%) (Fig. 4a). The group of proteins with isomerase activity included DNA topoisomerase, rpiB family of sugar-phosphatase isomerase and hypoxantine phosphoribosyltransferase (indicated as parE, rpiB and hpt, respectively) (Additional file 1: Table S1). Of these isomerases, rpiB and hpt are metabolic enzymes involved in the pentose phosphate pathway of Carbohydrate degradation and the Purine metabolism, respectively.

When proteins were classified according to their molecular functions, most of the proteins from both culture conditions were largely involved in catalytic activities: 38.9% in anaerobiosis (Fig. 4c) and 35.7% in aerobiosis

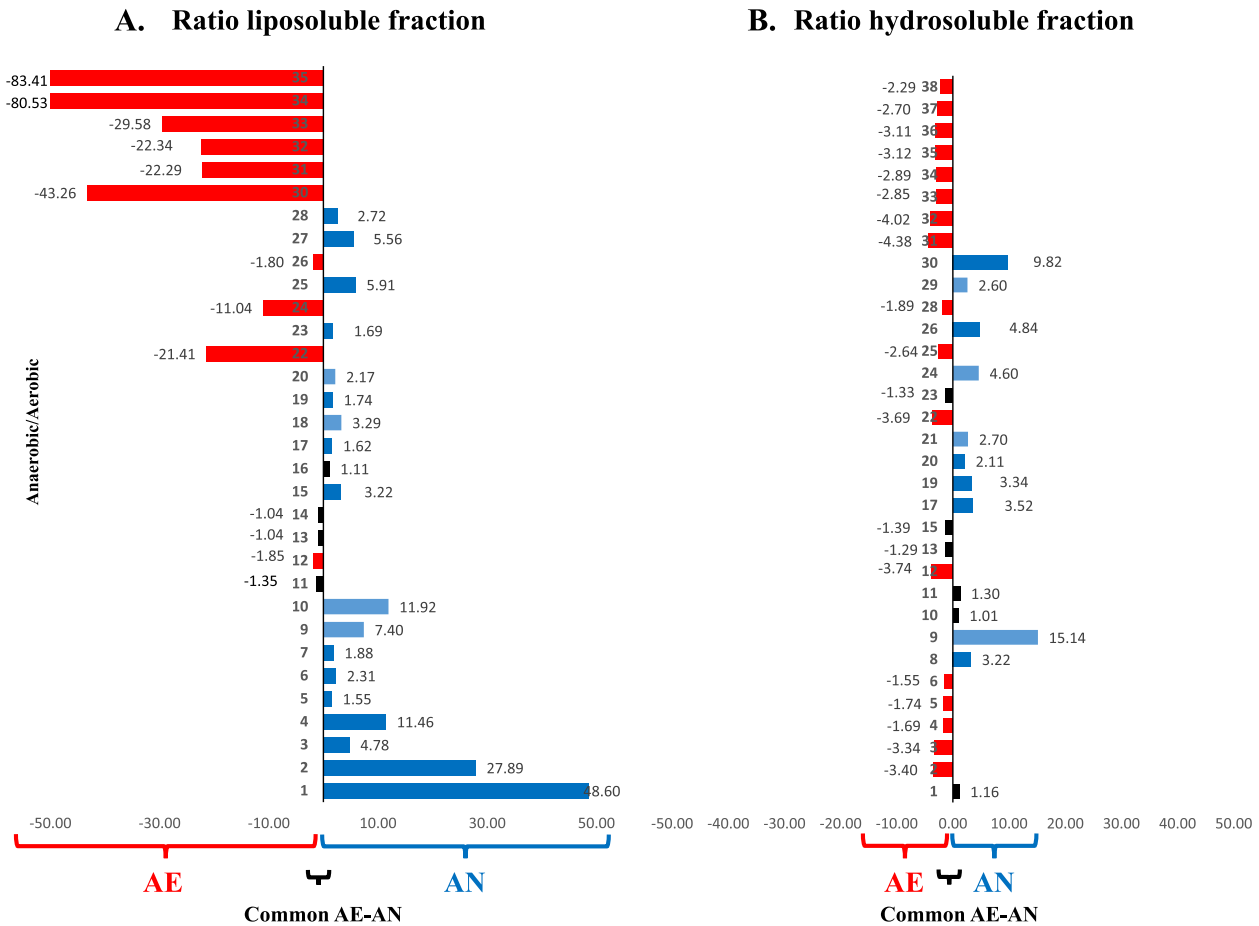

Fig. 3 Differentially expressed proteins in the liposoluble (**a**) and hydrosoluble (**b**) fractions obtained by 2D-Dige and MALDI-TOF/MS-MS analysis from *Mycoplasma fermentans* MFI grown in anaerobic conditions versus aerobic. The pick number from 1 to 35 for the liposoluble fraction and 1–38 for the hydrosoluble fraction, correspond to the proteins listed in Tables 1 and 2, respectively. A cut-off value of ±1.5 was used to distinguish between proteins similarly expressed in aerobic and anaerobic conditions (– 1.5 ≤ ratio ≤ + 1.5) from proteins comparatively more expressed (ratio > + 1.5 and < – 1.5). When the same protein was found in both fractions, only the spot with higher ratio, where the protein was most represented, was included. In red are shown the proteins overexpressed in the fraction from aerobic condition, in blue are the proteins overexpressed in the fraction from anaerobic condition and in black are the proteins commonly found in both aerobic and anaerobic

(Fig. 4d) (Additional file 1: Table S1). The amount of proteins with translation regulatory activity was higher in anaerobic mycoplasma (16.7% versus 7.1% in aerobic), while the proteins with structural activity were expressed at higher percentage in the aerobic mycoplasma (14.3% versus 5.6% in anaerobic) (Fig. 4c).

Despite the similarity in protein class and function observed from the PANTHER output (Fig. 4), different sets of proteins were specifically expressed in anaerobic as well in aerobic MFI (Additional file 1: Table S1).

Biological association among proteins

A major limitation of the PANTHER analysis was that numerous proteins from *Mycoplasma fermentans* were not found in the reference genome of *Mycoplasma genitalium* (see legend Additional file 1: Table S1). As a consequence, we were unable to predict metabolic pathways with PANTHER. Despite this limitation, we searched the STRING database [50] for the biological associations and network of the proteins belonging to the two different culture conditions (anaerobic and aerobic). *Mycoplasma hominis* was used as the reference genome. A protein–protein interaction network of the identified proteins is shown in Fig. 5. Table 4 indicates the biological processes, molecular function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of protein-protein interaction. The String database identified 4 KEGG pathways upregulated in the anaerobic mycoplasma and 3 KEGG pathways in the aerobic growth (Table 4). Specifically, we found that the culture of anaerobic MFI was enriched in metabolic pathways, biosynthesis of secondary metabolites and the RNA polymerase pathways. In contrast, the ribosome and the mismatch repair pathways were enriched in the aerobic

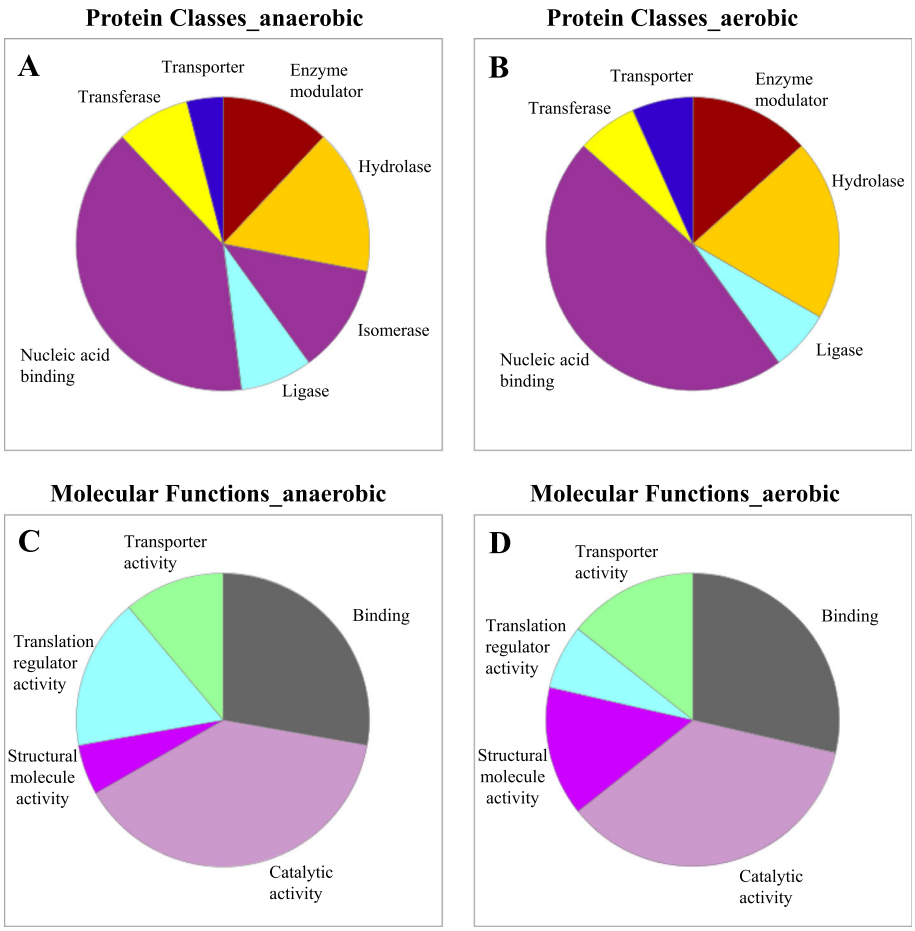

Fig. 4 The proteins identified in the fractions from *Mycoplasma fermentans* MFI cultured in aerobic and anaerobic conditions were analyzed using PANTHER software and classified according to protein class (**a**: anaerobic, **b**: aerobic) and molecular functions (**c**: anaerobic fraction, **d**: aerobic). *Mycoplasma genitalium* has been used as a reference genome for these classifications. In the analysis, PANTHER software did not include the following proteins: malL, asnA, xfp, argF, arcA, pmpr, hysA, abca, licA (anaerobic) and had, kdul, ndt, abca, licA, pcrA, rlmb. hysA (aerobic)

MFI. These results suggests that compared to the mycoplasma grown in aerobic conditions, various metabolic pathways are upregulated by mycoplasma in the absence of oxygen, while the purine metabolism was upregulated in both growth conditions.

Some proteins from anaerobic MFI, not comprised in the STRING analysis, were manually searched in Uniprot (https://www.uniprot.org) and included: argF that is part of the biosynthesis of secondary metabolites, MaIL and xfp that are enzymes involved in carbohydrate metabolic process, pmpr that is a transcriptional regulator, and the protein component of high-affinity transport system p37. Of note, p37 lipoprotein from *Mycoplasma hyorhinis* has the ability to promote cancer cell invasiveness and metastasis [52] and in our system it was overexpressed in anaerobic conditions (Table 3 and Fig. 3).

The proteins from aerobic MFI not comprised in the STRING analysis included: had, a DNA replication factor, kdui factor involved in glycan metabolism, pstb transport protein and rlmb, involved in RNA metabolism. Finally, proteins commonly expressed in MFI aerobic and anaerobic not found in STRING included: hisA, involved in carbohydrate metabolic process, nrna nuclease and the transport proteins abca and ptsL.

Discussion

Mycoplasmas are prokaryotic organisms of the class Mollicutes, widely spread in nature [2]. Because of their limited biosynthetic capabilities, mycoplasmas rely on the environment or host cells for some of their essential metabolic requirements, and can also grow as intracellular parasites exhibiting strict host and tissue specificities [21, 32].

Some mycoplasmas have been associated with human cancers, including prostate cancer [53], oral cell carcinoma [54] and non-Hodgkin's lymphoma (NHL) in HIV-seropositive subjects [55]. Although their role remains unclear and controversial, it has been shown that

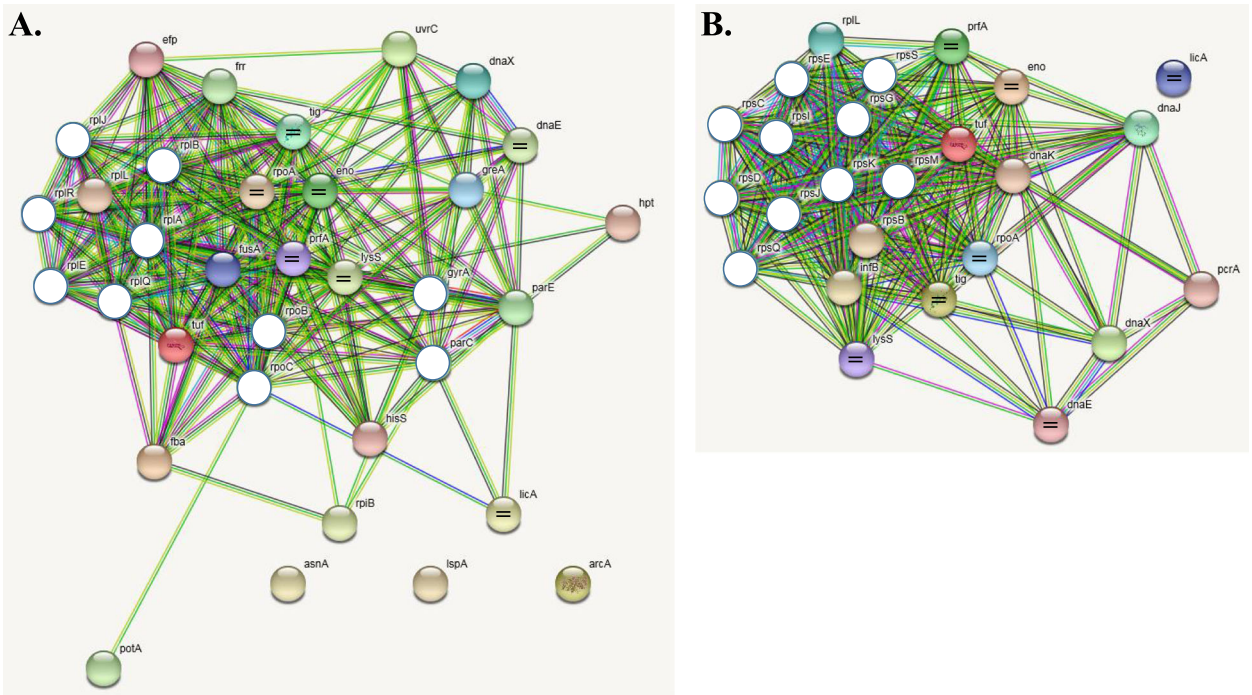

Fig. 5 The protein-protein interaction network analysis tool (STRING database) was used to analyze the proteins identified in the fractions of *Mycoplasma fermentans* MFI cultured in anaerobic (**a**) and aerobic (**b**) conditions. A network was build based on medium confidence (0.4) evidence. Proteins are indicated by nodes labeled with the gene symbol. The network was enriched in interactions, using the intersection of 10 additional proteins that were labeled as white nodes in the figure. *Mycoplasma hominis* was used as reference genome. The proteins labeled with the symbol '=' are present both in anaerobiosis and aerobiosis. **a**: represents the fraction of MFI cultured in anaerobic conditions showing 246 interactions between individual proteins (or nodes). In the analysis, STRING did not include the following proteins: argF, malL, xfp, p37 and pmpr (exclusive of anaerobic MFI) abca, ptsl, pdhA, nrnA and hysA (commonly present in both anaerobic and aerobic MFI). **b**: shows the fraction of MFI cultured in aerobic conditions depicting 212 interactions between individual proteins. In the analysis, STRING did not include the following proteins: had, kdul, ndt, rlmb and pstb (exclusive of aerobic MFI), abca, ptsl, pdhA, nrnA, and hysA (commonly present in both anaerobic and aerobic MFI)

infection with *Mycoplasma fermentans* subtype incognitus induces chromosomal alterations in vitro that result in phenotypic changes leading to acquisition of malignant properties in mouse and human cells, including loss of anchorage dependency, ability to form colonies in soft agar, and tumorigenicity in nude mice [56–58]. Infections with several mycoplasmas (fermentans, arginini, hominis and arthritidis) inhibit p53 activity and cooperate with Ras in oncogenic transformation in vitro, though the responsible bacterial protein has not been identified [59]. Also, mycoplasmas infections dysregulate gene expression profiles and post-translation modification in several cell lines [60]. These findings indicate that, in some cases, mycoplasmas could facilitate tumorigenesis, though (as mentioned above) no direct carcinogenic role for any mycoplasmas has been demonstrated in vivo. We isolated and characterized a strain of human mycoplasma able to induce lymphoma in a Severe Combined Immuno-Deficient (SCID) mouse model, consistent with a previously described lymphomagenesis dependent upon reduced p53 activity [40]. We demonstrated that this mycoplasma's DnaK, belonging to the HSP70 chaperone family, binds to human PARP1 and reduces its catalytic activity. PARP1 activates and recruits to the site of DNA damage important components of the DNA-repair complex. Moreover, this DnaK also binds human USP10 (ubiquitin carboxyl-terminal hydrolase 10, an important regulator of p53 stability), reducing p53 stability and anticancer functions. This indicates that, in cells where the DnaK is present, PARP1 and p53 anti-cancer activities will be reduced, increasing the likelihood of DNA instability and consequent malignant transformation. Mycoplasma was abundantly detected early in infected mice, but only low copy numbers of mycoplasma DnaK DNA sequences were found in primary and secondary tumors, suggesting a "hit and run/hide" mechanism of transformation, in which the critical events have occurred previous to cancer detection [40].

In addition, mycoplasmas have been associated with infectious diseases and post-infection pathologies both in humans and animals, and frequently persist also as chronic, asymptomatic infections [24–27, 61], but their

Table 4 Characteristics of biological process, molecular function and KEGG pathways of protein–protein interaction of anaerobic and aerobic Mycoplasma Fermentans with STRING 10.0

A. MFI Anaerobic		
Biological Process (GO)		
Pathway ID	Pathway description	matching protein
GO.0044267	cellular protein metabolic process	arcA,lysS,rplB,rplQ,tuf
GO.1901566	organonitrogen compound biosynthetic process	arcA,lysS,rplB,rplQ,tuf
GO.0006412	translation	lysS,rplB,rplQ,tuf
GO.0044249	cellular biosynthetic process	arcA,lysS,rplB,rplQ,tuf
GO.0010467	gene expression	lysS,rplB,rplQ,tuf
GO.0008152	metabolic process	arcA,licA,lysS,rplB,rplQ,tuf
GO.0006520	cellular amino acid metabolic process	arcA,lysS
Molecular Function (GO)		
GO.0003674	molecular_function	arcA,licA,lysS,rplB,rplQ,tuf
GO.0003824	catalytic activity	arcA,licA,lysS,rplB,tuf
KEGG Pathways		
03020	RNA polymerase	rpoA,rpoB,rpoC
00230	Purine metabolism	dnaE,dnaX,hpt,rpoA,rpoB,rpoC
01110	Biosynthesis of secondary metabolites	arcA,asnA,eno,fba,hpt,rpiB
01100	Metabolic pathways	arcA,asnA,dnaE,dnaX,eno,fba,hpt,rpiB,rpoA,rpoB,rpoC
B. MFI Aerobic		
Biological Process (GO)		
Pathway ID	Pathway description	matching protein
GO:0044267	cellular protein metabolic process	dnaK,lysS,rpsS,tuf
GO:0008152	metabolic process	dnaK,licA,lysS,rpsS,tuf
GO:0006412	translation	lysS,rpsS,tuf
GO:0010467	gene expression	lysS,rpsS,tuf
Molecular Function (GO)		
GO:0003674	molecular_function	dnaK,licA,lysS,rpsS,tuf
GO:0003676	nucleic acid binding	lysS,rpsS,tuf
GO:0032550	purine ribonucleoside binding	dnaK,lysS,tuf
GO:0032555	purine ribonucleotide binding	dnaK,lysS,tuf
GO:0035639	purine ribonucleoside triphosphate binding	dnaK,lysS,tuf
GO:0097159	organic cyclic compound binding	dnaK,lysS,rpsS,tuf
GO:1901363	heterocyclic compound binding	dnaK,lysS,rpsS,tuf
GO:0005524	ATP binding	dnaK,lysS
GO:0003723	RNA binding	rpsS,tuf
KEGG Pathways		
03010	Ribosome	rplL, rpsB, rpsC, rpsD, rpsE, rpsG, rpsI, rpsJ, rpsK, rpsM, rspQ, rpsS
03430	Mismatch Repair	dnaX, dnaE, pcrA
00230	Purine metabolism	dnaE,dnaX, rpoA

pathogenic mechanism(s) are not completely clear yet. In this regard, it is well known that mycoplasma can evade the immune-response by modulating the immune-system through a series of mechanisms, including the interaction of membrane lipid proteins with monocyte/macrophages [32, 62, 63]. Moreover mycoplasmas, like other bacteria, can be aerobic or facultative anaerobic microorganisms [21]. The biological determinants linked to the ability of growing in anaerobic or aerobic conditions, which is usually associated with localized infections [33, 34], have

never been shown in *Mycoplasma fermentans*. It is thus of interest to better characterize the mycoplasma proteins expressed in two different growth settings mimicking aerobic and anaerobic conditions observed in vivo [64].

A number of studies have addressed the correlation between protein expression and pathogenic potential of mycoplasmas by using proteomic analysis [65] both in human (such as *Mycoplasma pneumoniae* [66, 67], *Mycoplasma genitalium* [68, 69] and *Mycoplasma fermentans* M64 [12]) and in animals (more in details *Mycoplasma mobile* that infects fishes [70], and *Mycoplasma hypopneumoniae* and *Mycoplasma flocculare* that are pathogenic for swines [71, 72]).

Our findings show a high protein ratio of anaerobic versus aerobic when the liposoluble fraction was compared to the hydrosoluble (Fig. 3). Most of the lipoproteins of mycoplasmas are believed to be exposed to the extracellular surface and have been characterized to have roles in adherence, transport of nutrients and enzymatic activity. In addition, mycoplasma lipoproteins can be related to virulence and antigenicity [13, 14]. While many of the predicted lipoproteins of mycoplasmas remain hypothetical, our results suggest that the higher ratio of lipoproteins in the anaerobic mycoplasma might be associated to a more pathogenic phenotype. An example is the observed higher ratio of the p37 lipoprotein in the anaerobic fraction (Table 3).

By using the PANTHER software, proteins from each of the two growing conditions were classified depending on protein class and molecular functions. According to the class protein classification, our results show that proteins with isomerase activity were only found in mycoplasma grown in anaerobic conditions (Fig. 4a-b). In addition, the molecular function classification indicated that the anaerobic mycoplasma is enriched in proteins with translation regulatory activity, while more proteins with structural activity were expressed in the aerobic mycoplasma (Fig. 4c-d). However, a major limitation of the PANTHER analysis was that numerous proteins from *Mycoplasma fermentans* were not found in the reference genome of *Mycoplasma genitalium* (see legend Additional file 1: Table S1). Nevertheless, we were able to predict metabolic pathways with the STRING database (Fig. 5 and Table 4) and show that mycoplasma's growing conditions affect specific metabolic pathways (Table 4). In fact, while the KEGG pathway of purine metabolism was upregulated in both growing conditions, the anaerobic MFI was enriched in metabolic pathways, biosynthesis of secondary metabolites and the RNA polymerase pathways. In contrast, the ribosome and the mismatch repair pathways were upregulated in the aerobic MFI.

Metabolic activities are important determinants for Mollicutes taxonomy, and a number of studies highlighted the metabolism of mycoplasmas by linking the enzymatic activities detected in the mollicutes (for a review see [73]) Our study provides a description of metabolic changes reflecting the ability of mycoplasma to "switch" between two opposite growing conditions, aerobic and anaerobic. This would allow the microorganism to survive and replicate, and in turn to enhance the establishment of a proinflammatory environment in the host.

It is important to consider that our method only display highly expressed proteins in the two growth conditions of MFI, therefore emphasizing upregulation of different pathways. Nevertheless, common metabolic pathways for MFI aerobic and anaerobic, such as glycolysis, are expected to be in place, as suggested by the presence of the enolase (eno) and pyruvate dehydrogenase (pdhA) in both isolates (Tables 1 and 2). Though we show important differences between aerobic and anaerobic Mycoplasma associated to metabolic requirements, further studies are needed to better characterize all the stages of these processes.

Conclusions

The identification and characterization of virulence factors is of upmost relevance to discover new targets for the development of diagnostic methods, therapeutic drugs, and vaccines. For example, studies of other microorganisms' proteasome have revealed the presence of proteins like adhesins, transporters, nucleases, bearing virulence-related functional domains. Overall, these previous studies indicate the necessity of further and more comprehensive comparative proteomic studies, to deeply investigate possible pathogenicity or virulence-related differences at the protein level.

Our proteomic analysis shows the occurrence of phenotypical changes in mycoplasma due to oxygen availability. Such changes, associated to specific metabolic processes and molecular functions, are likely to be important for the survival of the micro-organism in two different conditions (aerobic and anaerobic), and eventually to continuously induce proinflammatory cytokines and other metabolites harmful to the host. Further studies are needed to better characterize all the steps of these processes, which could be correlated to immune-evasion and pathogenesis [74, 75].

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s41231-019-0047-2.

Additional file 1: Table S1. List of proteins identified in the fractions of *Mycoplasma fermentans* MFI cultured in anaerobic and aerobic conditions classified according to the GO annotations for protein classes and molecular functions using PANTHER software.

Abbreviations

2-DE: two-dimensional gel electrophoresis; Ae: aerobic; An: anaerobic; GO: Gene Ontology; MFI: *Mycoplasma fermentans* MFI

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Authors' contributions
FB and SC designed and performed experiments, analyzed data, and wrote the manuscript; SK and FC helped to perform the experiments and analyzed data; HT collected and analyzed data; RCG and DZ contributed to the design of the experiments and to the writing of the manuscript. All authors read and approved the final manuscript.

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Competing interests
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