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The Secretome of *Mycoplasma capricolum* subsp. *capricolum* in Neutral and Acidic Media

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Abstract

Mycoplasmas have proven to be successful pathogens despite their minimal genomes in some species. Their simplistic nature makes them an important tool for understanding the genes involved in basic metabolic processes and pathogenesis. Of particular interest are the genes involved in mycoplasma evasion of host immune responses and survival within phagocytic cells. The proteins secreted by mycoplasmas in the acidic and hydrolytic environment of a macrophage may help to uncover important virulence factors of this group of prokaryotes.

This study examined the effect of pH on the secretome of *Mycoplasma capricolum*. The bacterium was cultured in protein-free media and the culture supernatant was investigated for proteins produced during growth. Two-dimensional gel electrophoresis followed by MALDI-TOF mass spectrometry of tryptic digests was used to identify the proteins in this fraction.

Under acidic conditions, a total of a 111 protein spots from the secretome of *M. capricolum* were visualized by silver staining. A lower number of spots were detected in the neutral culture supernatant (82 spots). Superimposition of the two 2D maps revealed 26 proteins unique to the secretome at acidic pH but identification of all spots was not possible. The identification of neutral proteins revealed the presence of 7 putative lipoproteins, zinc metalloprotease, three peptidases, inorganic pyrophosphatase, nicotinamidase/pyrazinamidase, and ribosomal protein S4, and a number of hypothetical proteins. Proteins identified in the acidic culture supernatant included hemolysin A, peptide methionine sulfoxide reductases, two peptidases, nucleoside kinase, one lipoprotein, and others. An API ZYM enzyme assay demonstrated that the spectrum of enzymes secreted at an acidic pH was similar to that at neutral pH, the activities of the acid phosphatase, the phosphohydrolase, the C8 esterase lipase and the alkaline phosphatase were enhanced. The three key secreted proteins prominent at acidic pH were the acid phosphatase, the hemolysin, and the gelatinase.

Keywords: Secretome; Mycoplasma; Capricolum; Proteomic; Acidic pH

Introduction

Members of the mycoplasmas are known to have some the smallest free-living prokaryotes. They are characterized by their small genomes (0.58 – 2.20 Mb) and lack of a cell wall [1]. The availability of complete genome sequences of several mycoplasmas provides a great opportunity to understand the minimal genome biology of these small bacteria [2]. Considerable advances have been made towards understanding the pathogenesis of mycoplasmas; of particular interest is the interaction of these pathogens with the immune system, macrophage activation, cytokine induction, superantigens, autoimmune manifestations, and the evasion of host immune responses. However, despite the wealth of new genetic information emerging from bacterial genome sequencing, many newly discovered genes remain functionally unclassified [3]. It is suggested that these mollicutes are phylogenetically related to Gram-positive bacteria whereby their genomes were reduced through multiple events of mutations. This implies that these simple bacteria are not found at the root of the phylogenetic tree but are more likely late evolutionary products [4]. Owing to their reduced genomes mycoplasmas have limited biosynthetic capabilities and therefore tend to take on a parasitic lifestyle in which they obtain nutrients and other metabolic building blocks such as amino acids, nucleotides, fatty acids, and sterols from their hosts [1]. Generally Mycoplasmas enter their host and multiply over long periods of time, slowly wearing down the host [1]. This is one reason perhaps why mycoplasmas have been linked to AIDS pathogenesis and to malignant transformation, chromosomal aberrations, the Gulf War Syndrome, and other unexplained and complex illnesses, including chronic fatigue syndrome, Crohn's disease, and various arthritis' [5]. These microorganisms have developed a variety of mechanisms in order to deal with host immune responses including; mimicry of host antigens, survival within phagocytic and non-phagocytic host cells and the generation of phenotypic plasticity [1]. One of the most important questions about the pathogenesis of mycoplasmas is how they cause damage to the host cells and to what extent is the damage clinically apparent in the host.

M. capricolum is the causative agent of caprine arthritis, mastitis, and respiratory disease [6]. The sophisticated antigenic variation of *M. capricolum* makes it difficult for the host immune system to produce effective antibodies to suppress the infection [6]. The accumulation of hydrolytic enzymes produced by this species also contributes to tissue damage during infection [6]. A better understanding of the pathogenesis of these bacterial infections may be the result of exposing the nature of virulence factors elaborated by these pathogens during growth. The secretome is of particular interest as it shows the potential for elaborating such virulence factors.

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We have undertaken this study to characterize the secretome of *M. capricolum* using two-dimensional electrophoresis and mass spectrometry of tryptic digests. The results were compared to bioinformatics analysis of the secretome and supported with enzymatic characterization. This study provides new information and adds to the somewhat limited data on the biology of *M. capricolum*. Response of this pathogen to acidic pH provides a hint as to the mechanism of adaptation during infection and following phagocytic uptake in the host.

Materials and Methods

Bioinformatic analysis

The sequenced genome of *M. capricolum* (Accession # NC_007633) was retrieved from the World Wide Web NCBI site (www.ncbi.nlm.nih.gov/). The genome was first analyzed using the in-house program ExProt [7]. The program was used to identify the secreted / exported proteins of gram positive bacteria. The proteins that were predicted as being secreted or exported were further analyzed using the program SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP/) [8]. The amino acid sequences of the Signal peptidase I proteins were processed through the SignalP 3.0 server, specified to analyze gram positive bacteria.

Growth

Complete growth media (Modified Eaton's Medium, MEM) was composed of 8 g Mycoplasma Broth Base (Viragen, Ontario), 3.2 g of dextrose phenol red combination, and 100 ml of donor horse serum (The Cell Culture Company, PAA, Ontario), 100 µg/ml ampicillin (Sigma-Aldrich, St. Louis, MO) and 400 ml of distilled water, for a total final volume of 500 ml. The growth medium was modified in order to exclusively study the secretome of *M. capricolum*. The protein content was removed from the growth media by filtration through an Amicon filter (Millipore Stirred Cell 8400) with a 10,000 kDa cut off limit [9]. The culture medium was inoculated with *Mycoplasma capricolum* subsp. *capricolum* (California Kid type strain; kindly provided by Dr. Pascal Sirand-Pugnet, INRA, France) incubated at 37°C for 48 hours under 5% CO_2. This culture was then spun down at 13,000 rpm for 30 minutes at 4°C (Beckman, GS-15R Centrifuge). The cell pellet was then resuspended and washed in phosphate buffered saline (PBS) and centrifuged at 13,000 rpm for 30 minutes at 4°C. This pellet was split and resuspended in either 150 ml of Amicon Modified culture medium (AM) buffered with 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 5.5 (acidic) or 150 ml of AM buffered with MES at pH 7.5 (neutral). Culture supernatants were collected by centrifugation at 13,000 rpm for 30 minutes at 4°C followed by filtration through a 0.22 µm filter.

API ZYM enzyme assay

API ZYM (bioMerieux, Durham, NC, USA), a system designed for the detection of enzyme activities with certain chromogenic substrates, was used to determine the presence or absence of certain enzymes in the culture supernatants. Approximately 10 µg (65 µl) of proteins form the neutral and acidic protein sample was dispensed into each of the 20 cupules of the individual strips. The strips were then placed into their respective incubation boxes with 5 ml of distilled water. After inoculation, the plastic lid was placed on the incubation box and the box was incubated for 4 hours at 37°C. After the incubation period 20 µl of Fast Garnet GBC solution (5mls of 500 mM acetate buffer pH 6.2, and 2.5 mg/ml of Fast Garnet GBC salt) was added to each cupule on each strip and left exposed to daylight at room temperature for approximately 30 minutes until sufficient colour development had occurred.

Two-dimension SDS-PAGE

The volume of CFP used for isoelectric focussing was measured according to the protein concentration determined by the BCA protein assay. The immobilized pH gradient (IPG) strips (linear pH 3-10, 17-cm length, Bio-Rad) can accommodate up to 3 mg of protein while still maintaining proper protein resolution. Therefore volumes of CFP containing 3 mg of the Acidic CFP and 3mg of the neutral CFP were dried in the SpeedVac (DNA 120, Savant, Thermo Electron Corporation) for approximately 45 minutes at low heat. The dried CFPs were resuspended for 30 minutes in 350 µl of IPG rehydration buffer at room temperature (7M urea, 50mM dithiothreitol (DTT), 4% Chaps, 0.2% ampholytes, 0.001% bromophenol blue (BPB). The IPG strips were then rehydrated and isoelectric focussing (8-10,000V-hr at 20°C) was performed overnight (Protean IEF® Cell, Bio-Rad). IEF focusing conditions were the same for both the acidic and neutral CFPs. Once the first dimension was completed, the electrophoresed IPG strips were incubated for 20 minutes in equilibration buffer (6M urea, 0.375 M Tris HCl pH8.8, 2% sodium dodecyl sulphate (SDS), 20% glycerol) containing 2% DTT followed by and additional 20 minutes in equilibration buffer containing 2.5% iodoacetamide. Samples were subsequently subjected to the second dimension on a 10% separating gel (4% stacking) at a constant voltage of 200 V, and at 16 mA/gel for 30 minutes and 25 mA/gel for 5 hours using a vertical gel electrophoresis system by Whatman, Inc. Model V16-2 (Florham, NJ).

Silver staining

Proteins spots on the 17 cm 2D gels were visualized by silver staining methods (compatible with Mass Spectrometry analysis) based on the work of Blum et al. [10]. The gels underwent fixing in 40% methanol and 10% acetic acid. Two washes of 30% ethanol followed for 20 minutes each. A single wash of distilled H_2O followed for 20 minutes. The sensitizer containing 0.02% DTT was then applied to the gels for 10 minutes followed by three washes of distilled H_2O for 20 seconds each. The gels were placed in 0.2% silver nitrate for 20 minutes. Three washes of distilled H_2O followed for 20 seconds each. The gels were developed with 2% sodium carbonate and 0.04% Formaldehyde until the spots became visible. Three washes of distilled H_2O followed for 20 seconds each. The gels were placed in stop reagent containing 0.5% glycine overnight.

Mass spectrometry and protein identification

Protein spots of interest were manually excised from the 2D-gels and placed in siliconized sterile 1.5 ml microfuge tubes. In gel digestion and peptide extraction was based on a protocol provided by the manufacturer of the silver staining kit (Promega Inc.) with a modification of the protocol of Shevchenko et al. [11]. The protein spots were first destained by mixing in 50 µl of 50 mM ammonium bicarbonate, leaving them to sit for 5 minutes, and the wash solution was discarded as waste. The spots were then shrunk by the addition of 50 µl of 50% acetonitrile/25 mM ammonium bicarbonate, again the spots were vortexed and left to sit for 10 minutes; the liquid was discarded as waste. The spots were then reduced by the addition of 30 µl of 10 mM DTT solution for 30 minutes and incubation at 56°C, the supernatant liquid was discarded as waste. The spots were alkylated next by the addition of 30 µl of iodoacetamide for 15 minutes in the dark at room temperature. The supernatant liquid was again removed and discarded as waste. The protein spots were then shrunk again by the addition of 50 µl of 50% acetonitrile/25 mM ammonium bicarbonate. The mix was vortexed and left to stand for 15 minutes, the supernatant was discarded as waste. The protein spots were then digested with

trypsin. Each spot was covered with 13 ng/µl of trypsin, and the gel pieces were incubated overnight at 37°C. The tubes were removed from incubation and the liquid surrounding the gel pieces was transferred to a corresponding fresh sterile 500 µl microfuge tube. The tryptic peptides were sequentially extracted with 20 µl of each of the following: 25 mM ammonium bicarbonate, 5% trifluoroacetic acid (TFA), and 20 µl of 100% acetonitrile. The extracted peptides were dried in the SpeedVac for approximately 2.5 hours to decrease the volume from approximately 100 µl to 10 µl. Samples were subjected to MALDI-TOF Mass spectrometry (North Eastern Ontario Regional Cancer Center, Sudbury, Ontario). Proteins were identified using the Profound [12] server (http://prowl.rockefeller.edu/prowl-cgi/profound.exe).

Results

Bioinformatic analysis of the putative secretome of *M. capricolum*

Using the in-house program ExProt the entire predicted proteome of *M. capricolum* was treated for identifying proteins with signal sequences similar to Type I (secretory) or Type II (lipoproteins) signal sequences of Gram positive bacteria. Out of the 812 proteins from the *M. capricolum* genome (Accession # NC_007633) 247 of the proteins were identified as having either of these two signal sequences. Members of this set were then individually processed through the SignalP server which confirmed 60 lipoproteins (signal peptidase II) and 55 non-lipoproteins (signal peptidase I) within this set (Tables 1 and 2). This "filtered" set of proteins constitutes the core secretome and makes up about 14% of the total theoretical proteome of this pathogen.

API ZYM enzyme profiling

Culture supernatants were profiled for enzyme activities to compare with the findings of the bioinformatics and experimental protein identifications. This enzyme assay system provides qualitative evaluation of 20 enzyme activities. Culture supernatants of *M. capricolum* displayed similar enzyme activity profiles with 7 positive out of a total of 20 activities screened in this test (Table 3). These were the acid and alkaline phosphatases, C4 and C8 esterases/lipases, phosphohydrolase, β-galactosidase, and β-glucuronidase. There were distinct differences between the two culture supernatants however where the CFP from the acidic media showed higher activity for 4 of those enzymes: the acid and alkaline phosphatase, the C8 esterase/lipase, and the phosphohydrolase. This CFP also showed activity for 2 additional enzymes not detected in the neutral CFP: leucine arylamidase and α-chymotrypsin.

Proteomic analysis of culture supernatants

It was generally observed that the growth of *M. capricolum* in the acidic medium was retarded compared to that at neutral pH. It was also noted that the protein content of the acidic culture filtrate was consistently less compared to that at neutral pH. Representative maps of the 2D gels are presented in Figures 1 and 2. A total of 82 gel spots can be resolved from the 2D map of the neutral CFP (Figure 1) and 111 spots were recorded for the acidic CFP (Figure 2) using silver staining. Superimposition of both maps (not shown) revealed that 18 spots were unique to the neutral CFP which are numbered 4, 15, 23, 37, 58, 59, 60, 61, 62, 63, 70, 72, 74, 77, 78, and 82. The composite image also showed 26 spots that were unique to the acidic CFP and included spot numbers 83-108. It is important to note here however that there is no information on whether these represent unique proteins or simply proteolytic fragments of others. A significant number of protein spots failed to provide usable tryptic peptide fingerprint and were thus not identified. From the neutral CFP gel, a total of 27 proteins were identified (Table 4) compared to 12 proteins for the acidic CFP gel (Table 5).

Discussion

Treatment of the theoretical proteome of *M. capricolum* with our in-house program ExProt resulted in a predicted secretome size of 247 proteins out of a total proteome size of 812 proteins. This fraction of the proteome (30.4%) is comparable to predicted secretomes of other mycoplasmas including *M. pneumonia* (37%) and *M. genitalium* (24%) predicted with the same program [7]. A value of 32% for the *M. pneumonia* secretome was reported using the LocateProtein program [7]. These secretome sizes fall in the high end of the spectrum for secretome sizes and inspection of the predicted secretome of *M. capricolum* reveals that this high size possibly due to the large number of predicted lipoproteins as part of the secretome. Over 7% of the proteome of *M. capricolum* could potentially represent lipoproteins (Table 2). Treatment of the proteome using the SignalP program

#	Locus Tag	Protein Name	Protein ID
1	MCAP_0017	ATP-dependent metalloprotease FtsH	YP_424015.1
2	MCAP_0018	hypothetical protein	YP_424016.1
3	MCAP_0019	hypothetical protein	YP_424017.1
4	MCAP_0095	hypothetical protein	YP_424089.1
5	MCAP_0108	hypothetical protein	YP_424100.1
6	MCAP_0115	hypothetical protein	YP_424107.1
7	MCAP_0116	oligopeptide ABC transporter, oligopeptide-binding protein, putative	YP_424108.1
8	MCAP_0135	hypothetical protein	YP_424126.1
9	MCAP_0165	oligopeptide ABC transporter, oligopeptide-binding protein, putative	YP_424155.1
10	MCAP_0168	hypothetical protein	YP_424158.1
11	MCAP_0200	spermidine/putrescine ABC transporter, permease protein and spermidine/putrescine-binding protein	YP_424187.1
12	MCAP_0203	50S ribosomal protein L20	YP_424190.1
13	MCAP_0204	50S ribosomal protein L35	YP_424191.1
14	MCAP_0240	hypothetical protein	YP_424227.1
15	MCAP_0256	Oxidoreductase, short chain dehydrogenase/reductase family	YP_424242.1
16	MCAP_0265	hypothetical protein	YP_424251.1
17	MCAP_0270	ABC transporter, permease protein, putative	YP_424256.1
18	MCAP_0271	hypothetical protein	YP_424257.1
19	MCAP_0299	hypothetical protein	YP_424285.1
20	MCAP_0305	hypothetical protein	YP_424291.1

21	MCAP_0328	hypothetical protein	YP_424313.1
22	MCAP_0345	hypothetical protein	YP_424329.1
23	MCAP_0347	hypothetical protein	YP_424331.1
24	MCAP_0349	hypothetical protein	YP_424333.1
25	MCAP_0351	hypothetical protein	YP_424335.1
26	MCAP_0357	hypothetical protein	YP_424341.1
27	MCAP_0360	hypothetical protein	YP_424344.1
28	MCAP_0361	hypothetical protein	YP_424345.1
29	MCAP_0362	hypothetical protein	YP_424346.1
30	MCAP_0381	hypothetical protein	YP_424361.1
31	MCAP_0386	50S ribosomal protein L32	YP_424366.1
32	MCAP_0398	hypothetical protein	YP_424378.1
33	MCAP_0399	hypothetical protein	YP_424379.1
34	MCAP_0401	hypothetical protein	YP_424381.1
35	MCAP_0435	hypothetical protein	YP_424409.1
36	MCAP_0444	hypothetical protein	YP_424418.1
37	MCAP_0511	hypothetical protein	YP_424484.1
38	MCAP_0522	hypothetical protein	YP_424494.1
39	MCAP_0547	30S ribosomal protein S16	YP_424518.1
40	MCAP_0567	hypothetical protein	YP_424536.1
41	MCAP_0638	hypothetical protein	YP_424606.1
42	MCAP_0657	hypothetical protein	YP_424622.1
43	MCAP_0664	hypothetical protein	YP_424628.1
44	MCAP_0680	50S ribosomal protein L18	YP_424644.1
45	MCAP_0691	50S ribosomal protein L22	YP_424655.1
46	MCAP_0692	30S ribosomal protein S19	YP_424656.1
47	MCAP_0731	ABC transporter, substrate-binding protein	YP_424691.1
48	MCAP_0735	hypothetical protein	YP_424695.1
49	MCAP_0736	hypothetical protein	YP_424696.1
50	MCAP_0737	hypothetical protein	YP_424697.1
51	MCAP_0738	hypothetical protein	YP_424698.1
52	MCAP_0763	hypothetical protein	YP_424713.1
53	MCAP_0825	phosphonate ABC transporter, phosphonate-binding protein, putative	YP_424772.1
54	MCAP_0842	hypothetical protein	YP_424784.1
55	MCAP_0864	hypothetical protein	YP_424806.1

Table 1: Secreted / exported signal peptidase I proteins, predicted through the use of ExProt (ExProt) and SignalP 3.0 server.

#	Name of Proteins	Locus tag	Protein ID
1	lipoprotein, putative	MCAP_0033	YP_424031.1
2	lipoprotein, putative	MCAP_0034	YP_424032.1
3	lipoprotein, putative	MCAP_0042	YP_424039.1
4	lipoprotein, putative	MCAP_0059	YP_424055.1
5	lipoprotein, putative	MCAP_0072	YP_424067.1
6	lipoprotein, putative	MCAP_0172	YP_424162.1
7	lipoprotein, putative	MCAP_0211	YP_424198.1
8	lipoprotein, putative	MCAP_0212	YP_424199.1
9	lipoprotein, putative	MCAP_0231	YP_424218.1
10	lipoprotein, putative	MCAP_0242	YP_424229.1
11	lipoprotein, putative	MCAP_0268	YP_424254.1
12	lipoprotein, putative	MCAP_0269	YP_424255.1
13	lipoprotein, putative	MCAP_0272	YP_424258.1
14	lipoprotein, putative	MCAP_0273	YP_424259.1
15	lipoprotein, putative	MCAP_0287	YP_424273.1
16	lipoprotein, putative	MCAP_0312	YP_424298.1
17	lipoprotein, putative	MCAP_0329	YP_424314.1
18	lipoprotein, putative	MCAP_0346	YP_424330.1
19	lipoprotein, putative	MCAP_0348	YP_424332.1
20	lipoprotein, putative	MCAP_0350	YP_424334.1
21	lipoprotein, putative	MCAP_0352	YP_424336.1
22	lipoprotein, putative	MCAP_0409	YP_424384.1
23	lipoprotein, putative	MCAP_0431	YP_424405.1

24	lipoprotein, putative	MCAP_0432	YP_424406.1
25	lipoprotein, putative	MCAP_0433	YP_424407.1
26	lipoprotein, putative	MCAP_0442	YP_424416.1
27	lipoprotein, putative	MCAP_0451	YP_424425.1
28	lipoprotein, putative	MCAP_0470	YP_424444.1
29	lipoprotein, putative	MCAP_0471	YP_424445.1
30	lipoprotein, putative	MCAP_0513	YP_424485.1
31	lipoprotein, putative	MCAP_0514	YP_424486.1
32	lipoprotein, putative	MCAP_0524	YP_424496.1
33	lipoprotein, putative	MCAP_0525	YP_424497.1
34	lipoprotein, putative	MCAP_0526	YP_424498.1
35	lipoprotein, putative	MCAP_0561	YP_424530.1
36	lipoprotein, putative	MCAP_0583	YP_424552.1
37	lipoprotein, putative	MCAP_0585	YP_424554.1
38	lipoprotein, putative	MCAP_0584	YP_424553.1
39	lipoprotein (VmcD)	MCAP_0593	YP_424561.1
40	lipoprotein (VmcC)	MCAP_0594	YP_424562.1
41	lipoprotein (VmcB)	MCAP_0595	YP_424563.1
42	lipoprotein (VmcA)	MCAP_0596	YP_424564.1
43	lipoprotein, putative	MCAP_0605	YP_424573.1
44	lipoprotein, putative	MCAP_0607	YP_424575.1
45	lipoprotein (VmcE)	MCAP_0629	YP_424597.1
46	lipoprotein (VmcF)	MCAP_0630	YP_424598.1
47	lipoprotein, putative	MCAP_0648	YP_424614.1
48	lipoprotein, putative	MCAP_0661	YP_424625.1
49	lipoprotein, putative	MCAP_0704	YP_424664.1
50	lipoprotein, putative	MCAP_0720	YP_424680.1
51	lipoprotein, putative	MCAP_0721	YP_424681.1
52	lipoprotein, putative	MCAP_0723	YP_424683.1
53	lipoprotein, putative	MCAP_0727	YP_424687.1
54	lipoprotein, putative	MCAP_0758	YP_424709.1
55	lipoprotein, putative	MCAP_0770	YP_424719.1
56	lipoprotein, putative	MCAP_0782	YP_424731.1
57	lipoprotein, putative	MCAP_0799	YP_424747.1
58	lipoprotein, putative	MCAP_0801	YP_424749.1
59	lipoprotein, putative	MCAP_0843	YP_424785.1
60	lipoprotein, putative	MCAP_0846	YP_424788.1

Table 2: Secreted / exported Lipoproteins (signal peptidase II proteins) from Mycoplasma capricolum, predicted through the use of ExProt (ExProt).

Number	Enzyme	Substrate	Filtrate from neutral pH	Filtrate from acidic pH
1	CONTROL		-	-
2	Alkaline phospatase	2-naphthyl phosphate	+	++
3	Esterase (C 4)	2-naphthyl butyrate	+	+
4	Esterase Lipase (C 8)	2-naphthyl caprylate	+	++
5	Lipase (C 14)	2-naphthyl myristate	-	
6	Leucine arylamidase	L-leucly-2-naphthylamide	-	+
7	Valine arylamidase	L-valyl-2-naphthylamide	-	-
8	Cystine arylamidase	L-cystyl-2-naphthylamide	-	-
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	-	-
10	α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	-	+
11	Acid phosphatase	2-naphthyl phosphate	++	+++
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	+	++
13	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	-	-
14	β-galactosidase	2-naphthyl-αD-galactopyranoside	+	+
15	β-glucoronidase	Naphthol-AS-BI-βD-glucuronide	+	+
16	α-glucosidase	2-naphthyl-αD-glucopyranoside	-	-
17	β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside	-	-
18	N-acetyl-β-glucosamidase	1-naphthyl-N-acetyl-βD-glucosamide	-	-
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	-	-
20	α-fucosidase	2-naphthyl-αL-fucopyranoside	-	-

Table 3: Enzymatic profiling of the culture filtrates of *M. capricolum*.

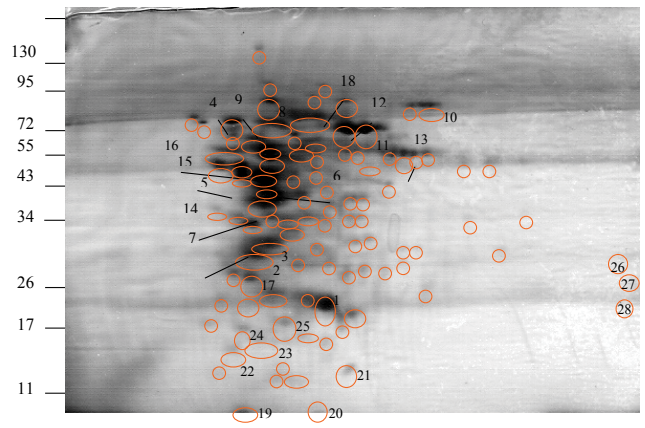

Figure 1: 2D-PAGE reference map of the culture filtrate proteins of neutral culture of *M. capricolum*.

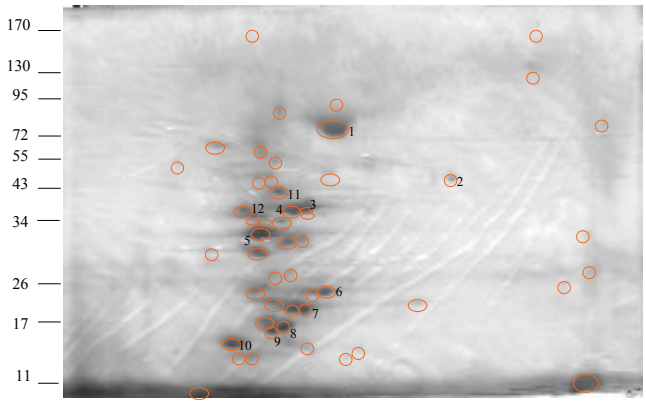

Figure 2: 2D-PAGE reference map of the culture filtrate proteins of acidic culture of *M. capricolum*.

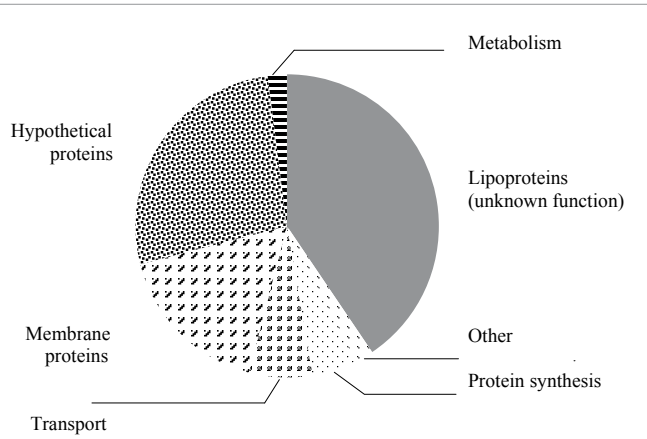

Figure 3: Functional grouping of the predicted secretome of *M. capricolum*.

reduced the secretome size from 247 proteins to 115, including the 60 predicted lipoproteins (Table 2). This reduces the secretome size from 30% to 14%. SignalP predicted 55 proteins with Type I signal sequence with a composition made up mostly of hypothetical proteins, transport proteins, and proteins involved in protein synthesis (Figure 3).

Enzymatic profiling of the culture supernatants of *M. capricolum* identified 7 enzyme activities in the neutral and acidic culture supernatants of *M. capricolum*. These were the acid and alkaline phosphatases, C4 and C8 esterases/lipases, phosphohydrolase, β-galactosidase, and β-glucuronidase. There were distinct differences between the two culture supernatants however where the CFP from the acidic media showed higher activity for 4 of those enzymes: the acid and alkaline phosphatase, the C8 esterase/lipase, and the phosphohydrolase. This CFP also showed activity for 2 additional enzymes not detected in the neutral CFP: leucine arylamidase and α-chymotrypsin. What was more curious however is the observation that the predicted secretome did not contain any proteins that could account for the observed enzymatic activities. This result was unusual and it's not clear why at this time. The ExProt and SignalP programs have been tested previously on many proteomes and their comparative prediction power was established many years ago [7]. If it is not the quality of the predictive programs then what the possible reasons for this observation. Cell lysis contribution as a possibility was looked at by measuring the levels of the cytoplasmic enzyme lactate dehydrogenase. Typical values for this activity in the CFP's used in this study were typically 10% that of the cytoplasmic extracts (data not shown). This activity is regularly used as part of the quality control of the CFP's used in our study. Other possible reasons may include for instance the dependence of *M. capricolum* on other secretory pathways to excrete proteins and that the general secretory pathway may only play a minor role in protein secretion in this bacterium. We should also not dismiss the fact that a large number of the predicted proteins in the secretome of *M. capricolum* have unknown functions (Tables 1 and 2). It is therefore possible that the observed enzymatic activities in the CFP's are in fact due to correctly predicted components of the secretome but have been annotated as having unknown functions due to lack of homologous proteins for sequence comparison.

The genomes of several mycoplasma species include distinctive sets of genes that encode different surface lipoproteins [13]. For the most part, it seems that these lipoproteins remain functionally unknown. However, some research suggests that surface lipoproteins confer both protective and opportunistic capabilities to *M. capricolum* based on analogous proteins. Lipoproteins of *M. hyorhinis* or *M. pulmonis* protect these pathogens from immunological targeting by host antibodies [14] and complement [15]. Additional functions for these surface proteins include the phase variation that is produced by mutations in certain gene clusters such as the variable *M. capricolum* subsp. *capricolum* (Vmc). The predicted secretome of *M. capricolum* contains the six Vmc (A-F) lipoproteins (Table 2). These genes and their products have been well characterized [13]. From the CFP of the *M. capricolum* grown at neutral pH a total of 28 proteins were identified by trypsin digest and MALDI-TOF mass spectrometry (Table 4). A major component of this fraction contained putative lipoproteins (8 out of 28, or 29%). The second major component of the CFP was made up of hypothetical proteins (5 out of 28, or 18%). The abundance of these two groups of proteins identified in this CFP matches that obtained through the bioinformatics analysis (Tables 1 and 2, Figure 3) where the lipoproteins and the hypothetical proteins represented the largest two fractions of the predicted secretome. A recent report by Couto et al. [16] outlined the identification of 19 proteins from the CFP fraction of *M. synoviae* cultured in modified Frey's medium. From the 19 identified proteins 3 were listed as hypothetical proteins. This is similar to our results were the hypothetical proteins made up 18% of the extracellular fraction. The authors did not report the presence of any

Spot	Locus Tag	Protein Name	Theo. Mass (Dalton)	Theo. pI	Peptide Match(es)
1	MCAP_0581	inorganic pyrophosphatase	21437.85	4.82	1179.7
2	MCAP_0064	UTP-glucose-1-phosphate uridylyltransferase	33187.53	5.37	829.5, 1021.6
3	MCAP_0309	conserved hypothetical protein	35583.32	5.21	862.2, 948.8, 1215.8
4	MCAP_0193	oligoendopeptidase F	70054.66	5.28	934.7, 1153.8, 1259.8, 1508.3, 1569.0, 1685.1, 1949.3, 1993.4
5	MCAP_0213	Enolase	49490.56	5.25	817.3, 878.2
6	MCAP_0490	dihydroxyacetone kinase 2 domain protein	61049.62	5.23	877.2, 861.2, 879.2, 2344.2
7	MCAP_0390	cell division protein FtsZ	40663.96	4.55	831.6, 845.7, 2226.3
8	MCAP_0583	lipoprotein, putative	86704.75	5.48	829.6, 861.2, 877.2, 917.7, 1021.7, 1151.7, 1233.6, 1242.8, 1332.9, 1435.9, 1772.2, 2053.4, 2511.3
9	MCAP_0193	oligoendopeptidase F	70054.66	5.28	934.7, 1153.8, 1259.8, 1508.3, 1569.0, 1685.1, 1949.3, 1993.4
10	MCAP_0242	lipoprotein, putative	90183.47	8.85	809.3, 921.7, 935.7, 1006.8, 1065.8, 1181.9, 1303.9, 1526.1, 1481.0, 1714.1
11	MCAP_0610	Transketolase	72762.48	6.24	914.5, 1765.8, 2225.2
12	MCAP_0610	Transketolase	72762.48	6.24	914.5, 1765.8, 2225.2
13	MCAP_0033	lipoprotein, putative (LppA)	60391.19	8.64	855.1, 878.1, 1569.0, 1684.1, 1993.3, 2186.2, 2400.2
14	MCAP_0420	dipeptidase, putative	50815.03	5.10	915.1, 963.6, 1074.6, 1375.8, 1507.0, 2064.0, 2311.2
15	MCAP_0127	cytosol aminopeptidase	49799.58	5.60	895.1, 933.6, 1088.1, 1274.7, 1700.0, 1701.1, 2221.2, 2190.1, 2705.3
16	MCAP_0042	lipoprotein, putative	61703.30	5.20	829.5, 861.2, 867.2, 1052.7, 1060.6, 1077.6, 1103.6, 1175.7, 1180.7, 1274.8, 1333.8, 1700.1, 1746.1, 2226.3,
17	MCAP_0194	conserved hypothetical protein	26372.97	4.71	849.5, 861.2, 993.6, 977.6, 1200.7, 2204.2
18	MCAP_0583	lipoprotein, putative	86704.75	5.48	829.5, 861.2, 877.1, 917.6, 1021.6, 1036.6, 1191.6, 1213.7, 1243.8, 1332.8, 1399.8, 1434.9, 1435.9, 1553.9, 1610.9, 1698.1, 1743.0, 1772.2, 2069.2, 2053.3, 2070.2, 2130.1, 2193.1, 2400.2
19	MCAP_0447	glycine cleavage system H protein, putative	13423.00	5.43	973.7, 1104.7, 1234.8, 1401.9, 1641.1, 1718.0, 1709.0, 1989.2, 2551.4, 2612.5
20	MCAP_0177	conserved hypothetical protein	14930.53	5.79	867.5, 1365.8, 1413.8, 1437.9, 1640.0, 1669.0, 1818.2
21	MCAP_0859	pyrazinamidase/nicotinamidase	18971.39	5.39	914.6, 895.6, 918.6, 1104.6, 1456.9, 1816.2, 2008.3, 2502.4
22	MCAP_0285	hypothetical protein	19142.74	4.60	956.3, 1109.7, 1186.7, 1729.1
23	MCAP_0308	conserved hypothetical protein	21383.15	4.31	867.5, 935.5, 1141.6, 2834.2
24	MCAP_0431	lipoprotein, putative	23800.42	4.70	828.5, 829.4, 891.4, 900.5, 931.5, 933.4, 973.5, 1588.8, 1874.8, 2744.3
25	MCAP_0433	lipoprotein, putative	24158.38	4.92	862.1, 878.1, 978.6, 1151.7, 1155.7, 1420.9, 2038.2, 2932.7
26	MCAP_0804	zinc metalloprotease, putative	27844.82	9.46	852.6, 914.6, 1041.6, 1126.7, 1112.7, 1288.8, 1610.9, 1640.1, 1850.1
27	MCAP_0763	membrane protein, putative	25949.18	9.43	831.5, 867.2, 966.6, 979.6, 1085.6, 1154.7, 1259.8, 1236.7, 1393.8, 2225.3, 2874.4, 2932.6
28	MCAP_0237	30S ribosomal protein S4	23870.91	10.08	927.6, 1002.6, 1163.7, 1223.7, 1254.7, 1308.8, 1428.9, 1841.1, 1919.0, 2189.2, 2195.2

Table 4: List of identified proteins from the culture filtrate of neutral culture of M. capricolum.

Spot	Locus Tag	Protein Name	Theo. Mass (Dalton)	Theo. pI	Peptide Match(es)
1	MCAP_0587	ABC transporter, ATP binding protein, putative	71588.27	6.34	808.7, 828.8, 851.8, 852.8, 877.4, 894.4, 907.9, 1088.4, 1266.1, 1597.4, 1676.3.
2	MCAP_0033	Lipoprotein, putative (LppA)	60391.19	8.64	847.5, 878.4, 915.3, 932.3, 1219.4, 1565.4.
3	MCAP_0195	Cytosol aminopeptidase	49831.5	5.90	863.5, 1300.7, 1332.8, 1332.1, 1332.5, 1421.2, 1421.4, 1597.8.
4	MCAP_0359	FoF_1 ATP synthase subunit beta	51302.8	5.10	861.4, 954.6, 1109.6, 1509.8, 1569.7.
5	MCAP_0722	Peptide methionine sulfoxide reductase (mrsA/ mrsB)	35732.66	5.46	898.8, 1279.1, 1288.1, 1793.3, 1905.7, 2164.8.
6	MCAP_0055	Hemolysin A	31160.50	9.14	831.7, 1003.8, 1586.1.
7	MCAP_0816	Hypothetical protein	25074.12	5.42	833.3, 877.3, 878.3, 909.3.
8	MCAP_0599	Nucleoside kinase	24310.95	5.80	992.4, 1126.4, 1133.7, 1834.6, 1922.7.
9	MCAP_0251	Transcription elongation factor GreA	17493.1	5.80	802.4, 858.4, 1069.6
10	MCAP_0175	Hypothetical protein	16409.8	4.80	1303.8, 1534.8,1878.9, 2386.2.
11	MCAP_0300	Hypothetical protein	57728.04	5.18	863.4, 878.4, 1274.6.
12	MCAP_0420	Dipeptidase, putative	50845.03	5.10	939.5, 996.9, 1192.9, 1235.4, 1359.1,

Table 5: List of identified proteins from the culture filtrate of acidic culture of M. capricolum.

lipoproteins in their findings. There have been several other proteomic studies of various mycoplasmas in the past few years but none of them focussed on the exported/secreted proteins [17-21]. Other notable findings in our study include the identification of several peptidases. A total of 4 different proteases were identified in 5 spots from the 2D gels (Table 4) to include oligoendopeptidase F (spots 4 and 9), dipeptidase,

cytosol aminopeptidase, and zinc metalloprotease. Gelatinases are metalloproteases and in our study gelatin zymography confirmed the presence of gelatinase activity in the CFP of *M. capricolum* (results not shown). The zinc metalloprotease identified in the CFP from the neutral culture medium (MCAP_0804) may be responsible for this activity. Aminopeptidases are exopeptidases that release N-terminal amino acid residues from their substrates; see review by Gonzales and Robert-Baudouy [22]. Aminopeptidase activity is well established in mycoplasma and is used in classification and identification of these microorganisms [23-25], including *M. capricolum* [26]. Although none of those studies looked at the secretome, aminopeptidases can be secreted and not only found in the cytosol or associated with the membrane or envelope in bacteria [22].

The 2D gel profile and the number of identified proteins of the CFP from the acidic media of *M. capricolum* were generally less complex than that from the neutral media. The bacterium did show reduced growth potential in acidic media and the reduced complexity of the CFP profile may be a reflection of this observation. A total of 12 proteins identified in this acidic CFP and its composition showed marked differences in comparison to that from neutral CFP. Of all the identified neutral and acidic proteins there were 3 that were seen in the secretome of *M. capricolum* at both a neutral and acidic pH. These included a putative lipoprotein (LppA), a putative dipeptidase, and a cytosol aminopeptidase. Only one lipoprotein in comparison was identified and the number of hypothetical proteins was 2. The acidic CFP also contained a putative hemolysin, a protein not identified in the neutral CFP. A previous study reported on the hemolytic activity of a number of mycoplasmas but not in *M. capricolum* [27]. One other protein of interest identified in the CFP from acidic growth media was the peptide methionine sulfoxide reductase (MCAP_0722; Table 5). This type of enzyme is typically involved in protecting bacteria from oxidative stress but a deletion mutant from *M. genitalium* was shown to exhibit reduced cytadherence and virulence [28].

Enzymatic profiling of both CFP's was evaluated to compare with the identification of the proteins in these preparations. The CFP from the neutral culture of *M. capricolum* contained a number of proteins that could explain some of the enzymatic activities observed in this protein fraction and includes the phosphatase, aryl amidase, and peptidase activities. No proteins were identified to explain the other enzymatic activities identified by the API ZYME system. Similarly, no proteins were identified in the acidic CFP that would explain those enzyme activities identified by the API ZYME system. The 2D gels however show a number of proteins for which the trypsin digestion and mass spectrometry failed to identify. What could possibly aid in the functional classification of the large number of functionally unknown proteins are the recent developments in the field of genome transplantation [29]. Gene knockout experiments are limited by the fact that gene function can be redundant and phenotypic changes associated with certain gene knockouts would thus not be observed to hint to a possible gene function. An additional limitation to these experiments is functional compensation where loss of a specific gene function is compensated for by up-regulation of other gene products. Genome transplantation on the other hand allows for deletion of large segments or gene clusters without causing fatal consequences to the recipient cell. The recent engineering of *Mycoplasma mycoides* JCVI [30] attests to the promise that this technique can be attainable and can provide a wealth of information on genome evolution and function.

Competing Interests

The authors declared that there are no competing interests.

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