Disruption of the S41 Peptidase Gene in *Mycoplasma mycoides capri* Impacts Proteome Profile, H$_2$O$_2$ Production, and Sensitivity to Heat Shock

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Abstract

Members of the *Mycoplasma mycoides* cluster are among the most virulent of the mycoplasmas, causing worldwide economically significant diseases of cattle and goats [1,2]. Two members of the cluster, *M. mycoides* subsp. *mycoides* Small Colony type and *M. capricolum* subsp. *capri* pneumonae (formerly F38 group), cause contagious bovine and caprine pleuropneumonia, respectively and are listed (http://www.selectagents.gov/selectagents and Toxins list.html) by USDA and APHIS (Federal Register 67, No. 155, 9 CFR 121.2b) and the World Organization for Animal Health (http://www.oie.int). Two closely related members of the *M. mycoides* cluster [3,4], *M. mycoides* subsp. *capri* (formerly *M. mycoides* subsp. *mycoides* Large Colony) and *M. capricolum*, are associated with respiratory disease in small ruminants worldwide and may also cause extrapulmonary complications and sepsis, but these pathogens are not listed agents.

Prior to the advent of genomic analysis, members of the *M. mycoides* cluster were speculated based on key phenotypic differences [5,6,7,8], including colony size on agar, biochemical activities, and thermal stability. A key phenotypic difference between the two species causing contagious pleuropneumonia and other members of the cluster, including *M. mycoides* subsp. *capri* GM12, is the ability to degrade casein [5,6,7], an activity that is quite uncommon in mycoplasmas. In addition to the differences in proteolytic activity, *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* SC also have different phenotypes with respect to carbohydrate utilization and thermal stability [8]. Further, production of H$_2$O$_2$ is a key virulence factor in the *M. mycoides* cluster [9,10,11,12,13,14,15], and quantitative differences in H$_2$O$_2$ production are directly linked to disease severity in mycoplasmas [9,10,13].

We identified the gene (MMCAP2_0241) responsible for the proteolytic phenotype in *M. mycoides* subsp. *capri* GM12 ATCC 35297 and created a mutant that has the coding sequence of the gene interrupted by insertion of TetA via homologous recombination [16]. *In silico* analysis of the predicted protein product of MMCAP2_0241 indicated a 651 amino acid protein with an unknown N-terminal domain (aa 25–339) and the S41 peptidase tail specific protease (TSP) domain (aa 340 to 544). Within the TSP domain, two carboxyl-tail processing (Ctp) motifs were found from aa 411–422 and aa 477–507. Also present in MMCAP2_0241 was a signal peptide domain (aa 1–24) and two transmembrane domains (aa 7–29 and 631–650). The alignment of MMCAP2_0241 showing conserved domains with other selected CtpA proteins is provided in Fig. S1, supplementary material. Because of the presence of the TSP domain, the presence of Ctp motifs, and homology with similar Ctp proteins in other bacterial species, we previously referred to this gene as ctpA [16].

Subsequently, we used the PHYRE (http://www.sbg.bio.ic.ac.uk/phyre2) automatic fold recognition server [17] to evaluate MMCAP2_0241. Based on this analysis, 266 residues (100% confidence, 41% coverage) modeled to the d1fc6a4 template for the superfamily ClpP, which also contains the TSP domain, two carboxyl-tail processing (Ctp) motifs were found from aa 411–422 and aa 477–507. Also present in MMCAP2_0241 was a signal peptide domain (aa 1–24) and two transmembrane domains (aa 7–29 and 631–650). The alignment of MMCAP2_0241 showing conserved domains with other selected CtpA proteins is provided in Fig. S1, supplementary material. Because of the presence of the TSP domain, the presence of Ctp motifs, and homology with similar Ctp proteins in other bacterial species, we previously referred to this gene as ctpA [16]. Subsequently, we used the PHYRE (http://www.sbg.bio.ic.ac.uk/phyre2) automatic fold recognition server [17] to evaluate MMCAP2_0241. Based on this analysis, 266 residues (100% confidence, 41% coverage) modeled to the d1fc6a4 template for the ClpP superfamily, which also contains the TSP domain, and is a carboxyl-tail processing S41 peptidase [18]. Because ClpP is known to encode a caseinolytic protease [18,19], we suggest that ClpP-like rather than CtpA may be the more appropriate designation. However, for consistency with the previous published
Pleiotropic Effects of M. m. capri S41 Peptidase
designation [16], we will refer to the mutant as the M. mycoides subsp. capri ctpA(clpP)::tetM mutant and to the protein as MMCAP2_0241 (ClpP-like).

Our M. mycoides subsp. capri ctpA(clpP)::tetM mutant, like the etiologic agents of contagious pleuropneumonia, lacks the proteolytic phenotype [0,16]. Therefore, it was of interest to determine if disruption of MMCAP2_0241 had significant impacts on the proteome profile of the microbe as well as other phenotypes that differentiate among members of the M. mycoides cluster [8]. Here we report that disruption of MMCAP2_0241 resulted in altered phenotypes reminiscent of M. mycoides subsp. mycoides SC. Specifically, the mutant exhibited increased sensitivity to heat shock as well as increased production of H$_2$O$_2$. We also observed decreased lactate dehydrogenase (LDH) activity and significant changes in the proteome profile in the M. mycoides subsp. capri ctpA(clpP)::tetM mutant.

Results
Disruption of MMCAP2_0241 resulted in increased sensitivity to heat shock and increased H$_2$O$_2$ production
As previously reported [16], the M. mycoides subsp. capri ctpA(clpP)::tetM mutant lost the proteolytic phenotype, as evidenced by lack of enzymatic activity on casein agar; MMCAP2_0241 gene disruption also was demonstrated by both Northern and Southern blots. With respect to carbohydrate use, the M. mycoides subsp. capri ctpA(clpP)::tetM mutant did not differ from the wild type with respect to fermentation of sorbitol or oxidation of maltose, trehalose, mannose or glucosamine (data not shown).

The disruption of MMCAP2_0241 altered the ability of the mutant to respond to heat stress (Fig. 1), resulting in a phenotype that was more similar to that of M. mycoides subsp. mycoides SC. At 37°C, the log CFU and growth rates of the wild type and M. mycoides subsp. capri ctpA(clpP)::tetM mutant were comparable at all time points (Fig. 1). However, after 3 hr at 42°C, the mutant grew more slowly than did the wild type strain (P<0.01). At subsequent time points, the difference was even more dramatic (P<0.001). The CFU of the wild type continued to increase, but the mutant failed to grow, indicating an increased sensitivity to heat shock. The M. mycoides subsp. capri ctpA(clpP)::tetM mutant exhibited a significant (P<0.01) increase in the production of H$_2$O$_2$ (Fig. 2), with the mutant producing approximately 40% more H$_2$O$_2$ than the wild type.

Disruption of MMCAP2_0241 had a significant impact on the proteome profile
In other microbes, deletion of ctpA or clpP is associated with pleiotropic effects and proteome differences [20,21,22,23]. Therefore, we next used two complementary approaches, 2D gel electrophoresis/differential gel electrophoresis (DIGE) and amine specific peptide-based labeling (iTRAQ$^\text{TM}$) followed by tandem mass spectrometry, to identify proteins that were perturbed in the M. mycoides subsp. capri ctpA(clpP)::tetM mutant. Both methods assess relative differences in the protein concentrations between the M. mycoides subsp. capri wild type and the M. mycoides subsp. capri ctpA(clpP)::tetM mutant. However, these two methodologies differ in that DIGE is protein-centric whereas iTRAQ is peptide-centric [24,25,26]. There are also discrepancies based on cellular compartmentalization (cytosol vs. membrane), abundance, and limitations inherent to each assay.

In our 2-D gels, eight proteins were found to differ significantly (P<0.006) between the M. mycoides subsp. capri wild type and M. mycoides subsp. capri ctpA(clpP)::tetM mutant (Table 1 and Fig. S2, supplementary material). Proteins that were significantly decreased in M. mycoides subsp. capri ctpA(clpP)::tetM were preprotein translocase SecA, adenylsuccinate synthase, phosphoglycerate kinase, and two conserved hypothetical proteins. The only proteins significantly increased in the mutant were the transcription termination protein NusG and serine-tRNA ligase. TetM was present only in the M. mycoides subsp. capri ctpA(clpP)::tetM mutant, as expected.

The iTRAQ$^\text{TM}$ analysis was more revealing than 2-D-DIGE analysis in that 221 proteins were identified with 95% confidence and an error factor <2 [27] (for complete dataset, see Tables S1 and S2 in supplementary material). Based on the Protein Pilot$^\text{TM}$ algorithm [27], 61 proteins were present in significantly changed concentrations (P<0.01) in the M. mycoides subsp. capri ctpA(clpP)::tetM mutant when compared to the wild type (Table 2; Table S2). In order to derive biological meaning from these differences, proteins were grouped according to global biologic functions as assigned in the Molligen 3.0 database [28] (http://cbi.labri.fr/ outils/molligen) or UNIPROT [29,30] (www.uniprot.org/).

An interesting feature in the M. mycoides subsp. capri ctpA(clpP)::tetM mutant is that most of the significantly altered proteins involved in metabolism were decreased, with the exception of putrescine carbamoyltransferase. Conversely, proteins that were significantly increased in the mutant are associated with genetic and environmental information processing. Proteins with an unassigned unknown function also tended to be decreased. Many of the proteins involved in glycolysis, including LDH, had reduced levels in the mutant. We chose to determine functional activity of

![Figure 1. Heat shock response in the Mycoplasma mycoides capri GM12 wild type and the ctpA(clpP)::tetM mutant.](image-url)
Figure 2. H$_2$O$_2$ production in the *Mycoplasma mycoides* capri GM12 wild type and the ctpA::tetM mutant. Production of H$_2$O$_2$ by 10$^8$ cells/ml of either the wild type or mutant was determined after a 30 min incubation with 100 μM glycerol. Production of H$_2$O$_2$ was significantly increased (P<0.01, unpaired T test) in the mutant. The standard reference curve is shown in the inset.

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Table 1. Proteins that significantly differed (P<0.006) in the *M. mycoides* subsp. *capri* ctpA::tetM mutant as determined by differential 2-dimensional gel electrophoresis.

| Spot | Accession   | Gene locus | Protein name                  | Prot. ID prob | Change  |
|------|-------------|------------|-------------------------------|---------------|---------|
| 1468 | GI:256383992| MMCAP2_0840| NusG                          | 99.9%         | Increase|
| 1471 | GI:256383992| MMCAP2_0840| Nus G                         | 100%          | Increase|
| 1472 | GI:256383992| MMCAP2_0840| Nus G                         | 100%          | Increase|
| 206  | GI:256383767| MMCAP2_0095| SecA                          | 100%          | Decrease|
| 1844 | GI:256384171| MMCAP2_0128| Hypothetical protein          | 99%           | Decrease|
| 602  | GI:256384387| MMCAP2_0061| Serine–tRNA ligase            | 100%          | Increase|
| 962  | GI:256384334| MMCAP2_0766| Adenylosuccinate synthase     | 100%          | Decrease|
| 1001 | GI:256383985| MMCAP2_0606| Phosphoglycerate kinase       | 100%          | Decrease|
| 123  | GI:256383953| MMCAP2_0480| Hypothetical protein          | 100%          | Decrease|
| 517  | GI:108795342| N/A        | TetM [E. coli]                | 100%          | Increase|

The spot numbers correspond to spot identifications on 2D gels (see Figure S1 in supplementary material). Accession numbers refer to GenBank (http://www.ncbi.nlm.nih.gov/genbank). The protein sequences are available at GenBank (http://www.ncbi.nlm.nih.gov/bioproject?term=PRJNA39245) and via Genomes on Line (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi).

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Table 2. Proteins that were significantly altered by the disruption of MMCAP2_0241 in Mycoplasma mycoides subsp. mycoides as determined by iTRAQ™.

| Gene Locus   | Protein Name/Product                                                                 | Change | Mean Ratio ± SD | Mean P value ± SD |
|--------------|--------------------------------------------------------------------------------------|--------|-----------------|-------------------|
| **Environment Information Processing: Membrane transport** |                                                                                   |        |                 |                   |
| MMCAP2_0247  | Phosphate ABC transporter, ATP-binding protein                                       | Inc    | 1.357±0.106     | 0.003±0.004       |
| MMCAP2_0336  | PTS system, IIa component                                                            | Dec    | 0.600±0.010     | <0.001±1.15E−05   |
| MMCAP2_0386  | Signal recognition particle protein (Fifty-four homolog)                             | Inc    | 1.487±0.0416    | <0.001±4.65E−04   |
| **Genetic Information Processing: Folding, Sorting and Degradation** |                                                                                   |        |                 |                   |
| MMCAP2_0165  | Thioredoxin                                                                          | Dec    | 0.640±0.010     | <0.001±7.51E−05   |
| MMCAP2_0213  | Phosphorpyruvate hydratase                                                           | Dec    | 0.603±0.021     | <0.001±5.77E−06   |
| MMCAP2_0256  | ATPase family associated with various cellular activities (AAA) protein              | Inc    | 1.373±0.049     | <0.001±0.001      |
| **Genetic Information Processing: Replication and Repair** |                                                                                   |        |                 |                   |
| MMCAP2_0771  | Ribonucleoside diphosphate reductase, alpha subunit                                  | Dec    | 0.710±0.0264    | <0.001±0.001      |
| MMCAP2_0773  | Ribonucleotide diphosphate reductase, beta subunit                                   | Dec    | 0.643±0.021     | <0.001±2.00E−05   |
| MMCAP2_0885  | tRNA uridine 5-carboxymethylaminomethyl modification enzyme                          | Inc    | 1.550±0.095     | 0.003±0.004       |
| **Genetic Information Processing: Transcription** |                                                                                   |        |                 |                   |
| MMCAP2_0407  | RNA polymerase sigma-A factor                                                        | Inc    | 1.396±0.038     | <0.001±1.50E−04   |
| **Genetic Information Processing: Translation** |                                                                                   |        |                 |                   |
| MMCAP2_0255  | 305 ribosomal protein S18                                                            | Inc    | 1.500±0.100     | <0.001±0.001      |
| MMCAP2_0661  | Serine-tRNA ligase                                                                   | Dec    | 0.630±0.060     | 0.003±0.002       |
| MMCAP2_0283  | 305 ribosomal protein S12                                                            | Inc    | 1.390±0.010     | 0.003±0.002       |
| MMCAP2_0284  | 305 ribosomal protein S7                                                             | Inc    | 1.337±0.080     | <0.001±1.15E−05   |
| MMCAP2_0151  | Translation elongation factor Tu                                                     | Inc    | 1.247±0.0321    | 0±0               |
| MMCAP2_0222  | Threonine-tRNA ligase                                                                | Dec    | 0.587±0.021     | <0.001±3.00E−05   |
| MMCAP2_0362  | 305 ribosomal protein S16                                                            | Inc    | 1.427±0.060     | 0.003±0.001       |
| MMCAP2_0159  | Isoleucyl-tRNA synthetase                                                            | Dec    | 0.657±0.040     | 0.003±0.002       |
| MMCAP2_0644  | 505 ribosomal protein L17                                                            | Inc    | 1.427±0.021     | <0.001±0.001      |
| MMCAP2_0653  | 505 ribosomal protein L15                                                            | Inc    | 1.457±0.035     | <0.001±1.73E−05   |
| MMCAP2_0656  | 505 ribosomal protein L6                                                             | Inc    | 1.417±0.055     | 0±0               |
| MMCAP2_0663  | 505 ribosomal protein L29                                                            | Inc    | 1.470±0.044     | 0.001±0.001       |
| MMCAP2_0667  | 305 ribosomal protein S19                                                            | Inc    | 1.397±0.015     | 0.003±0.002       |
| MMCAP2_0668  | 505 ribosomal protein L2                                                              | Inc    | 1.303±0.083     | <0.001±1.56E−04   |
| MMCAP2_0669  | 505 ribosomal protein L23                                                             | Inc    | 1.377±0.038     | 0.001±0.001       |
| MMCAP2_0671  | 505 ribosomal protein L3                                                              | Inc    | 1.310±0.036     | 0.001±3.96E−04    |
| MMCAP2_0672  | 305 ribosomal protein S10                                                             | Inc    | 1.467±0.0764    | 0.001±0.002       |
| MMCAP2_0680  | 505 ribosomal protein L7/L12                                                          | Inc    | 1.407±0.040     | 0±0               |
| MMCAP2_0689  | 505 ribosomal protein L1                                                              | Inc    | 1.417±0.051     | <0.001±3.01E−04   |
| MMCAP2_0810  | 505 ribosomal protein L11                                                             | Inc    | 1.507±0.027     | <0.001±2.25E−04   |
| **Metabolism: Amino acid** |                                                                                   |        |                 |                   |
| MMCAP2_0003  | D-lactate dehydrogenase                                                               | Dec    | 0.610±0.036     | 0±0               |
| MMCAP2_0059  | Alanine dehydrogenase                                                                  | Dec    | 0.680±0.020     | <0.001±5.77E−06   |
| MMCAP2_0120  | Threonine ammonia lyase                                                                | Dec    | 0.637±0.006     | 0.001±0.002       |
| MMCAP2_0235  | Pyruvate dehydrogenase (acetyl-transferring) E1 component, alpha subunit             | Dec    | 0.807±0.032     | <0.001±5.03E−05   |
| MMCAP2_0226  | Pyruvate dehydrogenase E1 component, beta subunit                                    | Dec    | 0.823±0.006     | <0.001±0.001      |
| MMCAP2_0519  | Isoleucyl-tRNA synthetase                                                            | Dec    | 0.657±0.040     | 0.003±0.002       |
| MMCAP2_0628  | Putrescine carboxamyltransferase                                                      | Inc    | 1.523±0.045     | <0.001±1.15E−05   |
| MMCAP2_0765  | Adenylsuccinate lyase                                                                  | Dec    | 0.560±0.044     | 0.002±0.002       |
| MMCAP2_0766  | Adenylsuccinate synthetase                                                            | Dec    | 0.450±0.036     | 0±0               |
| MMCAP2_0786  | Ornithine carboxamyltransferase                                                        | Dec    | 0.517±0.031     | 0±0               |
| **Metabolism: Other Amino Acids** |                                                                                   |        |                 |                   |
| MMCAP2_0059  | Alanine dehydrogenase                                                                  | Dec    | 0.680±0.020     | <0.001±5.77E−06   |
LDH because this enzyme is required to convert pyruvate to lactate, thereby recycling NADH. Because mycoplasma lacks the electron respiratory chain, recycling NADH is a key component to maintaining redox potential in the microbe. The *M. mycoides* subsp. *capri* ctpA (clpP)::tetM mutant exhibited about a 50% reduction in LDH activity in comparison to the wild type strain, *P*<0.0001 (Fig. 3). Thus, the observed functional activity was in agreement with the iTRAQ data for LDH.

### Table 2. Cont.

| Gene Locus       | Protein Name/Product                              | Change | Mean Ratio ± SD | Mean P value ± SD |
|------------------|--------------------------------------------------|--------|-----------------|-------------------|
| **Metabolism: Carbohydrate**                                                                 |
| MMCAP2_0036      | D-lactate dehydrogenase                           | Dec    | 0.610±0.036     | 0±0               |
| MMCAP2_0131      | Fructose-1,6-bisphosphate aldolase, class II      | Dec    | 0.667±0.064     | 0.002±0.003       |
| MMCAP2_0213      | Phosphopyruvate hydratase                         | Dec    | 0.603±0.021     | <0.001±5.77E−06   |
| MMCAP2_0225      | Pyruvate dehydrogenase (acetyl-transferring) E1 component, alpha subunit | Dec | 0.807±0.032 | <0.001±5.03E−05 |
| MMCAP2_0226      | Pyruvate dehydrogenase E1 component, beta subunit| Dec    | 0.823±0.006     | <0.001±0.001      |
| MMCAP2_0336      | PTS system, IIa component                         | Dec    | 0.600±0.010     | <0.001±1.15E−05   |
| MMCAP2_0451      | NADP-dependent glyceroldehyde-3-phosphate dehydrogenase | Dec | 0.560±0.010 | 0±0              |
| MMCAP2_0606      | Phosphoglycerate kinase                           | Dec    | 0.593±0.006     | 0±0               |
| MMCAP2_0607      | glyceroldehyde-3-phosphate dehydrogenase, type I | Dec  | 0.797±0.031     | 0±0               |
| MMCAP2_0733      | Phosphoglucomutase or phosphomannomutase          | Dec    | 0.717±0.0389    | 0.002±0.002       |
| MMCAP2_0831      | Ribose phosphate pyrophosphokinase                | Dec    | 0.653±0.0328    | <0.001±5.20E−05   |
| **Metabolism: Cofactors and Vitamins**                                                                 |
| MMCAP2_0464      | Lipote-protein ligase A                           | Dec    | 0.443±0.015     | 0±0               |
| **Metabolism: Energy**                                                                                                 |
| MMCAP2_0344      | Inorganic diphosphatase                           | Dec    | 0.760±0         | 0.002±0.002       |
| **Metabolism: Enzyme Families**                                                                                         |
| MMCAP2_0188      | Oligoendopeptidase F                             | Dec    | 0.750±0.023     | 0.002±0.004       |
| **Metabolism: Nucleotide**                                                                                             |
| MMCAP2_0129      | CTP synthase                                     | Dec    | 0.550±0.026     | <0.001±1.04E−04   |
| MMCAP2_0765      | Adenylosuccinate lyase                           | Dec    | 0.560±0.044     | 0.002±0.002       |
| MMCAP2_0766      | Adenylosuccinate synthetase                      | Dec    | 0.450±0.036     | 0±0               |
| MMCAP2_0771      | Ribonucleoside diphosphatase reductase, alpha subunit | Dec | 0.710±0.026 | <0.001±0.001 |
| MMCAP2_0773      | Ribonucleoside diphosphatase reductase, beta subunit | Dec | 0.643±0.021 | <0.001±2.00E−05 |
| MMCAP2_0831      | Ribose phosphate pyrophosphokinase               | Dec    | 0.653±0.032     | <0.001±5.20E−05   |
| **Metabolism: Lipid**                                                                                                   |
| MMCAP2_0218      | Glycerol kinase                                  | Dec    | 0.777±0.015     | <0.001±0.001      |
| **Unclassified**                                                                                                        |
| MMCAP2_0077      | Putative hydrolase of the HAD family             | Dec    | 0.720±0.028     | 0.001±4.01E−04    |
| MMCAP2_0189      | Conserved hypothetical protein                    | Dec    | 0.685±0.049     | <0.001±2.40E−04   |
| MMCAP2_0224      | Probable lipoate-protein ligase A                 | Dec    | 0.695±0.007     | 0.001±0.001       |
| MMCAP2_0237      | Dihydroxyacetone kinase, phosphotransfer subunit | Inc  | 2.040±0.056     | <0.001±1.15E−05   |
| MMCAP2_0440      | Putative lipoprotein                             | Dec    | 0.810±0.014     | 0.0017±0.002      |
| MMCAP2_0459      | Glycerol ABC transporter, glycerol binding protein| Dec | 0.455±0.007 | 0±0              |
| MMCAP2_0460      | Conserved hypothetical protein                    | Dec    | 0.815±0.007     | 0.001±0.002       |
| MMCAP2_0480      | Conserved hypothetical protein                    | Dec    | 0.725±0.035     | 0.001±0.001       |
| MMCAP2_0699      | Peptide methionine sulfoxide reductase            | Dec    | 0.375±0.007     | <0.001±3.18E−04   |
| MMCAP2_0700      | Putative lipoprotein                             | Dec    | 0.655±0.035     | 0.001±0.001       |

The gene locus was identified using the Molligen database and the *M. mycoides* subsp. *capri* GM12, taxon:436113 genome sequence. A protein may be listed in more than one category if it has multiple functions. Accession numbers refer to GenBank (http://www.ncbi.nlm.nih.gov/genbank). The protein sequences are available at GenBank (http://www.ncbi.nlm.nih.gov/bioproject?term = PRJNA39245) and via Genomes on Line (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi). Protein ratios were generated by dividing the spectral intensity of a specific protein in the *M. mycoides* subsp. *capri* ctpA (clpP)::tetM mutant by the spectral intensity of the specific protein in *M. mycoides* capri GM12. Protein ratios were calculated with the Pro Group™ algorithm (Applied Biosystems/MDS SCIEX). Only ratios from the spectra that are distinct to each protein (or protein form) were used for the calculation. Only proteins with 95% or greater confidence and an error factor (EF)<2 were considered. Proteins were decreased in the mutant if the mean ratio was <1.0; proteins were increased in the mutant if the mean ratio was >1.0. Proteins were grouped according to global biologic function as assigned in the Molligen 2.0 database [28] and/or UniProt [29,30]. Protein ratios were considered significantly different if they had P values <0.05 as determined by the Pro Group™ algorithm [27].

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acetone phosphate with the concomitant release of H$_2$O$_2$ [14,45]. Mycoplasmas, phosphorylated, and then metabolized to dihydroxyacetone, glycerol also can be used by many mycoplasmas restricted catabolic pathways, and many of the pathways observed [22,44].

Glycerol oxidation and the subsequent release of H$_2$O$_2$ plays a central role in the cytotoxicity of M. mycoides subsp. mycoides SC and the M. mycoides cluster [10,11,12,14,15] as well as in M. pneumoniae [9,46]. Increased production of H$_2$O$_2$ also has been associated with increased virulence and toxicity in other ruminant mycoplasmas, including M. agalactiae [13], M. arginini [47], M. bovis [13], and M. ovipneumoniae [47]. European strains of M. mycoides subsp. mycoides SC are significantly less virulent than African strains and have reduced H$_2$O$_2$ production [12,14,48]. Genomic analysis [14,48,49] has shown that the European strains lack an 8.3 Kb DNA segment in the gtsABC operon and are less efficient at glycerol uptake, thus explaining the reduced H$_2$O$_2$ production. A similar gtsABC operon was described in M. leachii [15], which produces levels of H$_2$O$_2$ comparable to African strains of M. mycoides subsp. mycoides SC. However, the production of H$_2$O$_2$ by itself is not enough to elicit the cytotoxic effect. For example, vaccine strains of M. mycoides subsp. mycoides SC are able to produce H$_2$O$_2$ comparable to virulent strains, but have attenuated virulence [10], suggesting that additional virulence factors are required. Based on in vitro studies with adhesin-deficient mutants [10,12], it is likely that strong contact between the mycoplasma and the host cell is required for H$_2$O$_2$ toxicity, and that this direct contact facilitates the translocation of H$_2$O$_2$ into the cytoplasm of the host cell. Thus, both adherence to the host cell surface as well as the release of H$_2$O$_2$ is required for toxicity.

The disruption of MMCAP2_0241 in M. mycoides subsp. capri impacted a number of proteins, including LDH. The M. mycoides subsp. capri ctpA(clpP)::tetM mutant had a significant reduction (about 50%) in LDH activity. Although the full biological impact of this reduced activity is not known, it could potentially contribute to oxidative stress. Under normal conditions, M. mycoides subsp. capri catalyzes sugars to pyruvate via glycolysis [11,45,50]. Pyruvate can be reduced to lactate by LDH, which results in reoxidation of NADH to yield NAD$^+$ [45]. Because these microbes do not possess an electron transport chain that can be used for this purpose, the recycling of NADH is critical to maintain cellular redox balance [51] and likely to be a crucial point for the adjustment of mycoplasmal metabolism [51,52].

Another potential source of oxidative stress is reactive oxygen species (ROS) like H$_2$O$_2$. We observed an approximately 40% relative increase in production of H$_2$O$_2$ in the mutant. Increased H$_2$O$_2$ may result in damage not only to the host but also to the microbe via oxidation of macromolecules like proteins and lipids. Although most mycoplasmas are deficient in superoxide dismutase and catalase [53], both thioredoxin (TrxA) and methionine sulfoxide reductase (Msr) are present and can modulate oxidative stress and ameliorate the toxic effects of ROS [53,54]. Interestingly, however, significant decreases as measured by iTRAQ$^TM$ were observed in the M. mycoides subsp. capri ctpA(clpP)::tetM mutant for both Msr (37% decrease) and Trx (65%) proteins, suggesting that the mutant might be under oxidative stress and less able to ameliorate the toxic effects of ROS. The observed increases in H$_2$O$_2$ levels could be the result of metabolic changes. Most notably, the twofold increase in dihydroxyacetone kinase in the ctpA(clpP)::tetM mutant and the decrease in several enzymes in the glycolytic pathway are suggestive of a shift to glycerol metabolism. Because catalase and peroxiredoxin are not present in the genome sequence of M. mycoides subsp. capri, it is highly unlikely that the increased levels of H$_2$O$_2$ we observed are due to a decrease in these activities.

The upregulation of ribosomal protein expression under stress conditions has previously been reported in mycoplasmas. The increased expression of ribosomal proteins may reflect the need to increase the translation and protein synthesis. Since the ctpA(clpP)::tetM mutant is more susceptible to heat shock and more...
exposed to oxidative stress, it may experience more protein damage and misfolding. This is consistent with the observed upregulation of ribosomal proteins in response to heat shock in both M. hypneumoniae [53] and M. pneumoniae [56]. Additionally, loss of the ctpA/clpP protein could impact the ability of the mutant to degrade damaged or aberrant proteins [35]. Thus, the increased translation could be a compensatory mechanism to cope with these consequences.

Our M. mycoides subsp. capri ctpA(clpP);tetM mutant, like the M. mycoides subsp. mycoides SC, was sensitive to heat shock [8]. Although the mechanism by which MMCAP2_0241 (ClpP-like) is involved in thermal stability in M. mycoides subsp. capri is unknown, it is interesting that disruption of an E. coli gene with a similar TSP-processing domain also resulted in sensitivity to high temperature [57], suggesting that the observed effect might be a result of loss of protein processing.

In our current study, the disruption of the MMCAP2_0241 gene clearly had a pleiotropic effect. Based on our results, it is reasonable to suggest that MMCAP2_0241 (ClpP-like) plays a role in stress response in M. mycoides subsp. capri. Although the specific mechanisms are not known and may be direct or indirect, it is important to note that by addressing changes in the proteome profile, we were able to identify proteins of interest that would not be predicted based on the loss of the proteolytic phenotype alone. In addition to the loss of the proteolytic phenotype, the mutant also exhibited reduced LDH activity, increased H$_2$O$_2$ production, and increased susceptibility to heat stress. The increased H$_2$O$_2$ production is particularly intriguing, as it may have implications for virulence in mycoplasmas [9,10,12,13,14,46,47] and also is a known source of oxidative stress in other bacteria [58].

Materials and Methods

Mycoplasma strains and cultivation

M. mycoides subsp. capri GM12 type ATCC 35297 wild type [1] and the M. mycoides subsp. capri ctpA(clpP);tetM mutant that was generated by double cross-over homologous recombination [16] were used in this study. M. mycoides subsp. capri GM12 type ATCC 35297 has been fully sequenced by The J. Craig Venter Institute, and the full sequences (both gene and protein) are available at GenBank (http://www.ncbi.nlm.nih.gov/ bioproject?term=PRJNA39245) and via Genomes on Line (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi). For all experiments, both strains were cultivated in parallel at 37°C in the same batch of SP4 medium, with the exception that the mutant was supplemented with 10$^12$ CFU per ml. All cultures contained 10$^9$ CFU per ml of growth of the mutant. Microbial growth was monitored by optical density at 640 nm, and cultures were harvested at late log phase (OD$_{640}$ = 0.08). All cultures contained 10$^9$ CFU per ml of medium, which was confirmed by direct colony counts. For proteomic and gel electrophoresis studies, cultures were concentrated by centrifugation and used at a final concentration of 10$^{12}$ CFU per ml.

Preparation of protein extracts for proteomics

Bacterial suspensions were divided into two aliquots: one aliquot was used for 2D gel electrophoresis and the second was used for iTRAQ™ followed by tandem mass spectrometry. Bacterial suspensions were pelleted by centrifugation and washed with wash buffer solution (Calbiochem ProteoExtract® kit, San Diego, CA). For 2D gel electrophoresis, proteins were extracted with Trizol (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocol. Pelleted protein extracts were allowed to air dry and were stored at −20°C. For iTRAQ™ analysis, proteins were extracted with ProteoExtract® Complete Mammalian Proteome Extraction Kit (Calbiochem, San Diego, CA) according to the manufacturer’s protocol. The total protein concentration of all samples was determined with the Non-Interfering Protein Assay™ Kit (Calbiochem, San Diego, CA).

2-Dimensional differential gel electrophoresis (2D-DIGE) and protein identification

Protein pellets were prepared for 2D electrophoresis as previously described [59]. Three 2-D electrophoresis experiments were performed with samples obtained from three independent experiments. The Cy2 internal standard was used to co-detect, match and normalize protein spots in all three gels. Gel images were obtained with Typhoon 9600 Variable Mode Imager (GE Healthcare, Piscataway, NJ) and images were analyzed with DeCyder 2D version 7.0 software (GE Healthcare, Piscataway, NJ). Protein ratios for each gel spot were generated by dividing the total area of M. mycoides subsp. capri ctpA(clpP);tetM spot by the total area of the corresponding wild type M. mycoides subsp. capri spot. Only protein ratios that were 2-fold or greater in difference were considered for further analysis. An automated spot picker (ProPiec Workstation, Digilab Genomic Solutions Inc., Ann Arbor, MI) selected protein targets for identification. The same protein spot from each biological replicate was pooled for processing and identification by tandem mass spectrometry as previously described [59]. Tandem mass spectrometric data was searched against NCBI nr bacterial database using Mascot (Matrix Science, Boston, MA) database search engine. Protein identification was performed with Scaffold version 2.01.02 (Proteome Software Inc., Portland, OR). Protein identifications were accepted if they could be established at greater than 80.0% probability and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [60]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Quantitative proteomics using peptide labeling and 2D-LC-MS/MS

In order to minimize variability, the protein extracts from all three biological replicates of wild type M. mycoides subsp. capri (control) were combined and the total protein concentration of the pooled sample was adjusted to match the total protein concentration of each biological replicate of M. mycoides subsp. capri ctpA(clpP);tetM mutant. Bacterial protein extracts were prepared as previously described [59] and labeled with an amine specific peptide-based labeling system, iTRAQ™, according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Labeled samples were analyzed inline with a hybrid quadrupole-TOF mass spectrometer QSTAR (Applied Biosystems Inc) as previously described [59]. Tandem mass spectra were extracted by Analyst (v 1.1.; Applied Biosystem Inc) and the NCBI bacterial protein database (concatenation of the forward and random sequences) was used for protein identification. Searches were performed using MS/MS data interpretation algorithms from Protein Pilot™ (Paragon™ algorithm, v 3.0, Applied Biosystem Inc) and Mascot (v 2.2, Matrix Science, London, UK) as already described [59]. Quantification of protein ratios was based on a minimum of three spectra. Only protein ratios with an error factor (EF) <2 were retained for further analysis. EF is a measure of the variation among the different iTRAQ™ ratios (the greater the variation, the greater the uncertainty) and represents the 95% uncertainty range for a reported ratio. Proteins were...
grouped according to global biologic function as assigned in the Molligen 2.0 database [28] and/or UniProt [29,30]. Protein ratios were considered significantly different if they had P values< 0.01 as determined by the Pro Group™ algorithm [27].

Carbohydrate use

We compared the carbohydrate use of M. mycoides subsp. capri and the ctpA(clpP):tetM mutant using the BBL Crystal™ system (Becton Dickinson Microbiology Systems, Cockeysville, Md) according to the manufacturer’s instructions. Both strains were grown to midlogarithmic growth in 2 ml of SP4 broth. Cells were pelleted, suspended in the inoculation medium, the kit test plate inoculated and incubated at 37°C for 24 hr before reading results. Tests were performed in triplicate.

Response to heat shock

Both wild type and mutant strains were grown to midlogarithmic phase at 37°C as described above. Each culture was divided into two aliquots. One aliquot was kept at 37°C and the other was immediately transferred to 4°C. Each treatment was done in triplicate. At selected time points, an aliquot was removed, serially diluted, and the CFU determined by direct colony count.

Measurement of H2O2 production

H2O2 production was determined as previously described for M. mycoides subsp. mycoides SC [10]. Six replicates were performed for each strain. Briefly, wild type and mutant M. mycoides subsp. capri were grown to midlogarithmic phase and harvested by centrifugation at 10,000 rpm for 15 min at 4°C. Bacterial pellets were washed 3 times in incubation buffer (67.7 mM HEPES, pH 7.3; 140 mM NaCl, and 7 mM MgCl2). After the final wash, bacteria were resuspended in incubation buffer to a cell density of 108 cells/ml and incubated for 20 min at 37°C. Bacterial pellets, suspended in the inoculation medium, the kit test plate inoculated and incubated at 37°C for 24 hr before reading results.

Tests were performed in triplicate.

Measurement of lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity and concentration were measured using the Lactate Dehydrogenase Colorimetric Assay Kit (Abcam Inc., Cambridge, MA). Five replicates were performed for each strain. Briefly, 4 ml of both wild type and mutant M. mycoides subsp. capri were grown to midlogarithmic phase (106 cells/ml) and harvested by centrifugation as described above. The cell pellets were homogenized in 0.5 ml of cold assay buffer, centrifuged at 10,000 rpm for 15 minutes at 4°C, and the supernatant collected. Twenty µl of the supernatant was used in a total 200 µl reaction. A standard curve was constructed, and the LDH activity and concentration were measured and calculated according to the manufacturer instructions.

Statistical analysis

The 2D gel data, H2O2 production data, LDH activity, and heat shock growth data were analyzed by unpaired students T test or ANOVA. CFU data was log transformed prior to analysis by ANOVA. For statistical analysis of iTRAQ™ data, protein ratios were generated with Pro Group™ algorithm and automatically corrected for bias. The calculated P-value obtained with the ProGroup™ algorithm is based on 95% confidence interval. A P value<0.05 was accepted as significant.

Supporting Information

Figure S1 Alignment and conserved signature sequenc- es of M. mycoides subsp. capri (MMCAP2_0241) and M. capricolum (MCA 0240) compared with representative bacterial CTP proteins. Bacillus subtilis (ZP_03591706.1), Bartonella bacilliformis (YP_988644.1), Burkholderia pseudomallei (YP_107067.1), Escherichia coli (ZP_256314.1), Legionella pneumo- philia (YP_122899.1), Neisseria gonorrhoeae (ZP_04720812.1), Syne- cocus sp. (NP_898059.1), and Staphylococcus aureus (BAB12513.1) were used for comparative purposes. Identical and similar amino acid (aa) residues are marked red and blue, respectively. For M. mycoides capri, note the signal peptide domain (aa 1–24) followed by the N-terminal domain (aa 25–339), two transmembrane domains (aa 7–29 and aa 631–650) and the S41 peptide tail specific protease (TSP) domain (aa 340–544). Within the TSP domain and denoted by yellow boxes are the two carboxyl-tail processing (Ctp) motifs (aa 411–422 and aa 477–507). Protein sequences were aligned using Clustal Omega (http://www.clustal.org/omega) followed by analysis using the Sequence Manipulation suite: multiple align show (www.bioinformatics.org/oms). Highly con- served, identical amino acids within the TSP domain are highlighted in red; highly similar amino acids are highlighted in blue. Note that both the amino- and carboxyl- terminal portions of the proteins are quite diverse. (TIFF)

Figure S2 Differential 2-dimensional electrophoresis of M. mycoides subsp. capri GM12 wild type and M. mycoides subsp. capri ctpA (clpP):tetM mutant. A. The distribution of proteins in the wild type (Cy3 label, red) and mutant (Cy5 label, green). B. The distribution of protein spots that were significantly increased in the mutant. C. The distribution of protein spots that were significantly increased in M. mycoides capri. (TIFF)

Table S1 Proteins that were not significantly altered by the disruption of ctpA in Mycoplasma mycoides subsp. capri GM12. The gene locus and gene name, if annotated, were identified using the Molligen database and the M. mycoides subsp. capri GM12, taxon:436113 genome sequence. GI accession numbers refer to GenBank (http://www.ncbi.nlm.nih.gov/genbank). The protein sequences are available at GenBank (http://www.ncbi.nlm.nih.gov/bioproject?term = PRJNA39245) and via Genomes on Line (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi). Data is shown for three independent biological replicates. Protein ratios were calculated by dividing the spectral intensity of a specific protein in the M. mycoides subsp. capri ctpA (clpP):tetM mutant by the spectral intensity of the specific protein in M. mycoides subsp. capri GM12. Protein ratios were calculated with the Pro Group™ algorithm (Applied Biosystems/ MDS SCIEX). Only ratios from the spectra that are distinct to each protein (or protein form) were used for the calculation. The total protein score is the measurement of all the peptide evidence for a protein and is analogous to protein scores reported by other protein identification software. Each identified peptide within a protein was assigned a score based on confidence (95% confidence = 2, 95% confidence = 1.5). Raw peptide identification was performed using the Paragon™ database searching algorithm (Applied Biosystems/MDS SCIEX). Raw peptide identification was further processed with the Pro Group Algorithm™ (Applied Biosystems/MDS SCIEX). Only proteins with 95% or greater confidence and an EF factor <2 were considered. Proteins were decreased in the mutant if the ratio was <1.0; proteins were increased in the mutant if the ratio was >1.0. Proteins were
considered to beunchanged in the mutant if the P value was >0.001. Further, significance levels had to be obtained in all 3 biological replicates.

(XLSX)

Table S2 Proteins that were significantly altered by the disruption of ctpA in M. mycoides subsp. capri GM12. The gene locus and gene name, if annotated, were identified using the Molligen database and the M. mycoides subsp. capri GM12, taxon:436113 genome sequence. GI accession numbers refer to GenBank (http://www.ncbi.nlm.nih.gov/Genbank). The protein sequences are available at GenBank (http://www.ncbi.nlm.nih.gov/GenBank?term=PRJNA39245) and via Genomes on Line (http://www.genomeshareonline.org/cgi-bin/GOLD/index.cgi). Data is shown for three independent biological replicates. Protein ratios were generated by dividing the spectral intensity of a specific protein in the M. mycoides subsp. capri ctpA (clpP)::tetM mutant by the spectral intensity of the specific protein in M. mycoides subsp. capri GM12. Protein ratios were calculated with the Pro Group™ algorithm (Applied Biosystems/MDS SCIEX). Only ratios from the spectra that are distinct to each protein (or protein form) were used for the calculation. The total protein score is the measurement of all the peptide evidence for a protein and is analogous to protein scores reported by other protein identification software. Each identified peptide within a protein was assigned a score based on confidence (95% confidence = 2. 95% confidence = 1.5). Raw peptide identification was performed using the Paragon™ database searching algorithm (Applied Biosystems/MDS SCIEX). Raw peptide identification was further processed with the Pro Group Algorithm™ (Applied Biosystems/MDS SCIEX). Only proteins with 95% or greater confidence and an EF factor <2 were considered. Proteins were decreased in the mutant if the ratio was <1.0; proteins were increased in the mutant if the ratio was >1.0. Proteins were significantly different in the mutant if the P value was <0.001.

Further, significance levels had to be obtained in all 3 biological replicates.

(XLSX)

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Author Contributions

Conceived and designed the experiments: ABA MB LR. Performed the experiments: ABA MB LR. Analyzed the data: ABA MB LR. Contributed reagents/materials/analysis tools: ABA MB LR. Wrote the paper: ABA MB LR.

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