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Proteases as Secreted Exoproteins in Mycoplasmas from Ruminant Lungs and Their Impact on Surface-Exposed Proteins

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ABSTRACT Many mycoplasma species are isolated from the ruminant lungs as either saprophytes or true pathogens. These wall-less bacteria possess a minimal genome and reduced metabolic capabilities. Accordingly, they rely heavily on their hosts for the supply of essential metabolites and, notably, peptides. Seven of 13 ruminant lung-associated *Mycoplasma* (sub)species were shown to possess caseinolytic activity when grown in rich media and assessed with a quantitative fluorescence test. For some species, this activity was detected in spent medium, an indication that proteases were secreted outside the mycoplasma cells. To identify these proteases, we incubated concentrated washed cell pellets in a defined medium and analyzed the supernatants by tandem mass spectrometry. Secreted-protease activity was detected mostly in the species belonging to the *Mycoplasma mycoides* cluster (MMC) and, to a lesser extent, in *Mycoplasma bovirhinis*. Analyzing a *Mycoplasma mycoides* subsp. *capri* strain, chosen as a model, we identified 35 expressed proteases among 55 predicted coding genes, of which 5 were preferentially found in the supernatant. Serine protease S41, acquired by horizontal gene transfer, was responsible for the caseinolytic activity, as demonstrated by zymography and mutant analysis. In an *M. capricolum* mutant, inactivation of the S41 protease resulted in marked modification of the expression or secretion of 17 predicted surface-exposed proteins. This is an indication that the S41 protease could have a role in posttranslational cleavage of surface-exposed proteins and ectodomain shedding, whose physiological impacts still need to be explored.

IMPORTANCE Few studies pertaining to proteases in ruminant mycoplasmas have been reported. Here, we focus on proteases that are secreted outside the mycoplasma cell using a mass spectrometry approach. The most striking result is the identification, within the *Mycoplasma mycoides* cluster, of a serine protease that is exclusively detected outside the mycoplasma cells and is responsible for casein digestion. This protease may also be involved in the posttranslational processing of surface proteins, as suggested by analysis of mutants showing a marked reduction in the secretion of extracellular proteins. By analogy, this finding may help increase understanding of the mechanisms underlying this ectodomain shedding in other mycoplasma species. The gene encoding this protease is likely to have been acquired via horizontal gene transfer from Gram-positive bacteria and sortase-associated surface proteases. Whether this protease and the associated ectodomain shedding are related to virulence has yet to be ascertained.

KEYWORDS *Mycoplasma*, posttranslational cleavage, exosecretion, proteases

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Bacteria belonging to the *Mycoplasma* genus can colonize many animal hosts. They are wall-less and have very small genomes, typically around 1,000 kbp, resulting from reductive evolution from low-G+C *Firmicutes*. Consequently, they depend on their host for the supply of cholesterol, amino acids, nucleotides, etc. They are often host specific, and many are pathogenic. Mycoplasmas colonizing ruminants are a good study model, as they comprise a huge diversity of species and include the type species of the genus, *Mycoplasma mycoides* subsp. *mycoides*. *M. mycoides* subsp. *mycoides* was the first mycoplasma to be isolated, in 1898 (1), and is the causative agent of contagious bovine pleuropneumonia, a disease notifiable to the World Organization for Animal Health (OIE). Like many other ruminant mycoplasmas, *M. mycoides* subsp. *mycoides* shows marked tissue tropism toward the respiratory tract, where it induces severe lesions. It therefore came somewhat as a surprise that no obvious virulence factors were identified when the entire *M. mycoides* subsp. *mycoides* genome was sequenced (2).

A decade after the genome was sequenced, Browning et al. illustrated that the complexity of mycoplasma pathogenesis is "predominantly attributable to the immunopathological response of the host to the persistence of these pathogens" (3). This suggested that any gene that is involved in optimal adhesion, efficient nutriment scavenging, immune evasion, or immunomodulation and that is not required for *in vitro* growth might be involved in virulence (3). In this general picture, H_2O_2 production was a notable exception, as it corresponds to one of the few cases of production of cytotoxic compounds by mycoplasmas (4). However, H_2O_2 may not be indispensable for strain virulence (5). Until recently, mycoplasma virulence studies have focused mainly on interactions between the surface of the bacterium and its host. It was clear that mycoplasma immunopathology was linked to an imbalanced immunological response leading to exacerbated inflammation. Extensive work was performed as early as 1971 (6) and recently (7) to attempt to decipher the immune responses of the hosts. However, there is also a body of work focusing on mycoplasma cell-associated pathogenesis. *Mycoplasma bovis*, for example, is able to display a wide array of variable surface antigens coded by variable surface lipoproteins (Vsps) (8). The *in vivo* variability of Vsps, together with immunological factors of the host, may contribute to mycoplasma persistence and immunomodulation (9, 10).

More recently, targeted proteolysis of surface antigens, coupled with variable cleavage efficiency, was identified as another mechanism participating in the diversification of surface-exposed antigens (11). In the porcine respiratory pathogen *Mycoplasma hyopneumoniae*, posttranslational processing is an important mechanism for creating cell surface diversity (12), as 35 surface-associated proteins were shown to be subject to endoproteolytic cleavage. These modifications, affecting adhesins, lipoproteins, or moonlighting proteins, are likely to expand the mycoplasma antigen repertoire of the mycoplasma cell surface. Proteolytic cleavage often enhances the binding of host molecules such as plasminogen, whose conversion into the serine protease plasmin may have an important impact on host tissues and immune effector molecules (13). *M. mycoides* subsp. *mycoides* and many other mycoplasma species also express a "mycoplasma immunoglobulin protease," together with a mycoplasma immunoglobulin binding protein (14). This two-protein system allows the cleavage of host immunoglobulins and may therefore play a key role in immune evasion by mycoplasmas. Proteolysis obviously plays an important role in the natural history of mycoplasma species. This has notably been studied in the porcine pathogen *M. hyopneumoniae*, in which endoproteases are responsible for ectodomain shedding, with profound impact on host-pathogen interactions. A surface protein, P159, is cleaved, and this processing event generates new surface protein diversity, as well as functional redundancy in glycosaminoglycan and cilium binding (15). In addition, aminopeptidase activity has been evidenced at the surface of this pathogen (16, 17).

Fewer data are available for other lung-pathogenic mycoplasmas. The aim of this study was to determine which proteases may be expressed in mycoplasmas colonizing ruminant lungs and if those proteases may be secreted outside the mycoplasma cell as

part of an exoproteome. Naturally, this study was beset with the same difficulties encountered in studying extracellular exopolysaccharides (18), i.e., the need for rich and complex growth media to ensure mycoplasma growth. A similar approach was therefore adopted, based on the incubation of washed, concentrated mycoplasma cells into a defined medium allowing these mycoplasma cells to maintain their metabolism for some time. Secreted exoproteins were then analyzed by phenotypic tests such as by assays of digestion of skimmed milk and fluorescent casein and by casein zymography as well as by tandem mass spectrometry (MS-MS). The main involvement of an extracellular protease was finally confirmed by the use of mutant strains from two species in which the mutation occurred in orthologous genes and for which the exported caseinolytic activity was abolished.

RESULTS

Protease activity of mollicutes found in ruminant lungs. The global peptidase activity of mollicutes found in the respiratory tract of ruminants was first estimated by a quantitative approach using whole cultures in complex medium and fluorescently labeled casein as a universal substrate. The caseinolytic activity of 28 *Mycoplasma* and 2 *Acholeplasma* strains, corresponding to species usually isolated from ruminant lungs (Table 1), was assessed using two independent stationary-phase cultures in modified Hayflick's medium (m-Hayflick). There was a high heterogeneity of results in comparisons of one species to another, while the results obtained with different strains within a (sub)species were usually homogeneous, with the notable exception of *Mycoplasma capricolum* subsp. *capripneumoniae*, *M. putrefaciens*, *M. ovipneumoniae*, and *M. bovirhinis*. The highest levels of caseinolytic activities were measured for members of the *Mycoplasma mycoides* cluster (MMC), with relative activity (RA) values ranging from 47% to 95%. *M. leachii* and *M. mycoides* subsp. *capri* strains yielded the highest values. *M. ovipneumoniae* and *M. bovirhinis* strains presented values within this high range, though certain strains and culture replicates showed lower values. In contrast, *M. putrefaciens*, which is phylogenetically closely related to the *Mycoplasma mycoides* cluster, displayed very low RA values, comparable to those of distantly related species such as *M. arginini* and *M. alkalescens* (RA, 4% to 29%). All other species provided intermediate values ranging from 23% to 74%. It is noteworthy that *M. agalactiae* showed lower values (RA, 23% to 30%) than its close relative *M. bovis* (RA, 41% to 49%). Strains from seven (sub)species, namely, *M. capricolum* subsp. *capripneumoniae*, *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *mycoides*, *M. mycoides* subsp. *capri*, *M. ovipneumoniae*, *M. bovirhinis*, and *M. bovis*, displaying high overall activity, were selected for analysis of extracellular proteolytic activity. All these species displayed tropism toward ruminant lungs. *M. arginini*, which showed low overall caseinolytic activity, was selected as a negative control. In spite of their high caseinolytic activity, *M. leachii* strains were not included in subsequent analyses because current clinical cases are scarce and most often associated with polyarthritis rather than with pneumonia.

Assessment of extracellular proteolytic activity. The extracellular caseinolytic activity of a strain from each of the 8 selected (sub)species was assessed on milk plates, where proteases diffusing in the agar generate a translucent ring around the culture (Fig. 1). A wide area of milk digestion was observed for *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum* strains, although the appearance differed between the two species. For *M. mycoides* subsp. *capri* strains, there was a sharp zone where the milk was completely digested and a wider zone with diffuse digestion. For all other strains, the digestion zone was diffuse with various diameters. *M. capricolum* subsp. *capripneumoniae* strains displayed a conspicuous zone of digestion despite the cultures on solid media being barely visible, while *M. mycoides* subsp. *mycoides* and *M. bovirhinis* strains induced only weak digestion. *M. bovis* and *M. ovipneumoniae* strains, as well as the negative-control strain of *M. arginini*, did not display any detectable activity (data not shown). This phenotypic test clearly suggests that *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, and, to a much lesser extent, *M. mycoides* subsp. *mycoides* and *M. bovirhinis*, are all able to produce extra-

TABLE 1 Caseinolytic activity in mycoplasma cultures from species that can be isolated from ruminant lungs[a]

Phylogenetic group	Species and subspecies	Main host	Disease/clinical signs	Strain	Isolation yr	Country of origin or source (reference)	% overall caseinolytic activity[b]	
							Assay 1	Assay 2
Spiroplasma	***Mycoplasma capricolum* subsp. *capripneumoniae***	Goat	Contagious caprine pleuropneumonia	16125	2016	United Arab Emirates	85	63
				9231 Abomsa	1982	Ethiopia	47	58
	Mycoplasma capricolum* subsp. *capricolum	Goat	Contagious agalactia	Ck^T	1955	United States	82	90
				Ck-mut	2010	Allam et al. (22)	**40**	**45**
				94157	1994	Ethiopia	87	79
	Mycoplasma leachii	Cattle	Pneumonia	PG50T	1963	Australia	86	91
				ML06049	2005	Nigeria	95	95
	Mycoplasma mycoides* subsp. *mycoides	Cattle	Contagious bovine pleuropneumonia	Gladysdale	1953	Australia	64	71
				16113	2016	Namibia	64	72
				Rita	1987	Cameroon	60	68
				Rita-mut	2006	Unpublished data from CIRAD	**22**	**26**
				$PG1^T$	<1931	NK	48	59
	Mycoplasma mycoides* subsp. *capri	Goat	Contagious agalactia	GM12	1983	United States	92	89
				95010	1995	France	75	75
	Mycoplasma putrefaciens	Goat	Contagious agalactia	$KS1^T$	~1954	United States	12	4
				9231b	1992	France	11	29
Hominis	*Mycoplasma alkalescens*	Cattle	Pneumonia arthritis mastitis otitis	$PG51^T$	1961	Australia	7	4
				5561	2005	Nigeria	8	5
	Mycoplasma arginini	Ruminants	Opportunistic	Pontaumur	~1970	France	20	17
				Tizi ouzou	~1970	Algeria	13	12
	Mycoplasma ovipneumoniae	Sheep	Pneumonia conjunctivitis mastitis	9139-2/90	1991	Ethiopia	31	35
				$Y98^T$	~1971	Australia	63	59
				14811	2007	France	74	74
	Mycoplasma agalactiae	Goat	Contagious agalactiae	PG2T	1952	Spain	24	30
				94093-5633	1991	France	30	23
	Mycoplasma bovis	Cattle	Pneumonia mastitis	$PG45^T$	1962	United States	49	41
				Oger2	1975	France	45	45
	M. bovirhinis	Cattle	Commensal	MV5	~1970	France	49	69
				PG43	1965	England	57	32
				F11513	2017	France	58	53
Phytoplasma/ acholeplasma	*Acholeplasma laidlawii*	Environment	Commensal	$PG9^T$	<1963	NK	30	31
				PG8	<1963	NK	27	30

[a]A superscript "T" indicates the type strain. NK, not known. Data corresponding to species belonging to the *Mycoplasma mycoides* cluster are indicated with boldface. Mutant strain data are boldface and underlined.
[b]Overall caseinolytic activities are expressed as percent activity based on the logarithmic values corresponding to relative fluorescence unit (RFU) data measured in whole cultures in modified Hayflick's medium.

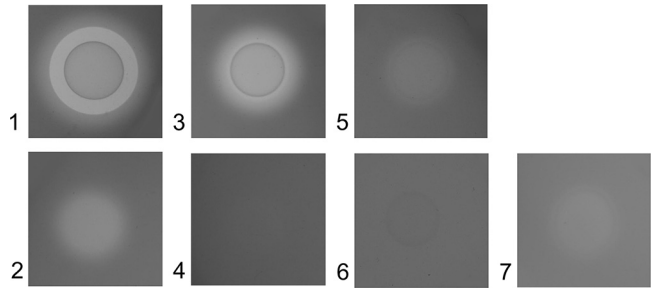

FIG 1 Casein digestion on an agar plate (modified Hayflick's medium) supplemented with 0.4% (wt/vol) milk. An area of casein digestion was observed for strains producing extracellular caseinolytic proteases. The strongest activity was observed for *M. mycoides* subsp. *capri* strain 95010 (panel 1), while a digested zone was evidenced for *M. capricolum* subsp. *capripneumoniae* Abomsa, although no conspicuous growth was observed for that fastidious strain (panel 2). The activity observed for *M. capricolum* subsp. *capricolum* Ck strain (panel 3) was completely abolished in the Ck-mut strain (panel 4) although its culture was clearly visible on the agar surface. Lower activity was evidenced for *M. mycoides* subsp. *mycoides* Rita (panel 5), and no activity was recorded for the mutated Rita-mut strain (panel 6). Slight activity was recorded for *M. bovirhinis* MV5 (panel 7).

cellular caseinolytic proteases released from the cell into the environment whereas *M. ovipneumoniae* and *M. bovis* are not. The extracellular caseinolytic activity of these strains was then confirmed by performing quantitative analysis using the fluorescently labeled casein assay in culture supernatants. The assay was first applied to cell pellets and supernatants from stationary-phase cultures in m-Hayflick medium (Table 2). The supernatants were filtered through 0.1-μm pores, and no living mycoplasma cells were found in the sample. All strains belonging to the *Mycoplasma mycoides* cluster (Table 1) exhibited higher caseinolytic activities in the supernatant than in the cell pellet. The opposite was found for the four other strains tested, including *M. bovirhinis*, which showed higher activities in the pellet. It is noteworthy that for *M. ovipneumoniae* and *M. arginini*, the activity was exclusively detected in the cell pellet. Under these experimental conditions, only the *Mycoplasma mycoides* cluster strains seemed to secrete proteases into the culture supernatant.

Extracellular caseinolytic activity in defined medium. Studies aiming at the characterization of extracellular components of mycoplasma cultures require the use of chemically defined media devoid of the complex supplements such as horse serum and yeast extract that are necessary for mycoplasma growth (18, 19). Here, a medium devoid of uncharacterized peptides, Opti-MEM, might facilitate extracellular protease identification. This medium was able to maintain mycoplasma viability during 16 h for the majority of the species tested, except for *M. arginini*, *M. ovipneumoniae*, and *M. bovis* (Table 2). Within the *Mycoplasma mycoides* cluster, *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri* still exhibited a very high level of caseinolytic activity in Opti-MEM supernatant, while the activity of *M. capricolum* subsp. *capripneumoniae* declined and that of *M. mycoides* subsp. *mycoides* completely disappeared. This was not due to a loss of viability, as the titers in cell suspensions remained stable until supernatant collection. In contrast to the observations made in rich medium, the caseinolytic activity of *M. bovirhinis* was higher in the supernatant after incubation in a chemically defined, serum-free medium. As in complex medium, none or negligible caseinolytic activity was detected for *M. arginini*, *M. bovis*, and *M. ovipneumoniae* supernatants in Opti-MEM (Table 2). However, the titers of viable cells in the Opti-MEM suspensions dropped sharply for those three species.

Identification of genes potentially coding for proteases in *Mycoplasma mycoides* subsp. *capri* and *Mycoplasma bovirhinis* species and tandem mass spectrometry analysis. As the greatest caseinolytic activity in culture supernatants was found in *M. mycoides* subsp. *capri* strains, the genome of strain 95010 was chosen as a model for the *Mycoplasma mycoides* cluster. Within this genome, 55 genes are predicted to potentially code for proteases (Table 3). Thirty-six were retrieved from the

TABLE 2 Caseinolytic activities of selected mycoplasma strains in modified Hayflick's or Opti-MEM medium, in pellet or supernatant

			Modified Hayflick's medium[b]				Opti-MEM medium[c]					
			% overall caseinolytic activity[d]									
Phylogenetic group	Species and subspecies	Strain[a]	Assay 1	Assay 2	% supernatant activity[d]	Cellular activity[d]	Casein digestion on agar plates[e]	Overall caseinolytic activity[d]	Supernatant activity[d]	Cellular activity[d]	T0 titer[f]	T16 titer[f]
Spiroplasma (*Mycoplasma mycoides* cluster)	*M. capricolum* subsp. *capripneumoniae*	9231 Abomsa	47	58	46	22	+	38	29	−3	9.3	9.3
	M. capricolum subsp. *capricolum*	Ck^T	82	90	85	43	+	90	69	24	9.9	10.0
		Ck-mut	**40**	**45**	**13**	**19**	**−**	**25**	**13**	**−1**	**9.0**	**9.3**
		94157	87	79	59	41	++	94	83	30	9.3	9.6
	M. mycoides subsp. *mycoides*	16113	64	72	54	26	+	27	1	2	8.7	9.8
		8740-Rita	60	68	56	30	+	11	3	−1	8.8	8.8
		Rita-mut	**22**	**26**	**5**	**24**	**−**	**7**	**−8**	**6**	**8.5**	**8.7**
	M. mycoides subsp. *capri*	95010	75	75	77	54	++	99	97	23	9.3	9.3
Hominis	*M. arginini*	Tizi Ouzou	13	12	2	29	−	13	−3	9	9.8	5.6
	M. ovipneumoniae	Y98^T	63	59	0	26	−	40	−5	9	8.9	6.7
	M. bovis	Oger2	45	45	9	48	−	25	3	2	8.9	7.6
	M. bovirhinis	MV5	49	69	29	76	+	50	49	20	9.6	9.6

[a]Strains chosen for extracellular activity research. Mutant strain names are in bold and underlined. Ck: California Kid.
[b]Culture in modified Hayflick's medium. Mutant data are in bold and underlined.
[c]Culture in supplemented Opti-MEM medium. Mutant data are in bold and underlined.
[d]Activity data are expressed in relative activity percentages.
[e]Casein digestion on milk agar plates was evaluated by measurement of the translucent area around bacterial spots and expressed in arbitrary units based on translucent area observations. −, no translucent area; +, blurred and small translucent area; ++, sharp and large translucent area; see Fig. S2.
[f]Titer (in $\log_{10}$ CFU per milliliter) at 0 h (T0) and after 16 h of incubation (T16) in supplemented Opti-MEM medium.

TABLE 3 Predicted protease-coding genes and tandem mass spectrometry detection of proteases for *M. mycoides* subsp. *capri* (strain 95010)[a]

Protein accession no.	Ratio × 10,000 (spectral count)		Fold change	Mnemonic	Gene name	Annotation (MEROPS and MaGe)
	Pellet (26,893)	Supernatant (11,964)				
CBW53764.1	50	2	0.0	**MLC_0360**	*ftsH*	FtsH-2 peptidase
CBW53797.1	0	0		MLC_0690		Family S9 unassigned peptidases
CBW53831.1	1	1		MLC_1030		Subfamily S41A nonpeptidase homologues
CBW53842.1	1	0		MLC_1140		Family S9 unassigned peptidases
CBW53849.1	42	14	0.3	**MLC_1210**	*pyrG*	CTP synthetase
CBW53854.1	4	0	0.0	MLC_1260		Subfamily S8A unassigned peptidases
CBW53874.1	141	317	0.2	**MLC_1460**	*pepA*	Family M17 unassigned peptidases
CBW53913.1	56	37	0.6	**MLC_1850**	*pepF*	Oligopeptidase F
CBW53915.1	84	195	2.3	**MLC_1870**	*pepA*	Family M17 unassigned peptidases
CBW53985.1	**0**	**397**	**397.0**	**MLC_2570**		**Subfamily S41A nonpeptidase homologues**
CBW54055.1	**0**	**38**	**38.4**	**MLC_3270**		**Subfamily S41A nonpeptidase homologues**
CBW54056.1	**3**	**53**	**13.2**	**MLC_3280**		**Subfamily S41A nonpeptidase homologues**
CBW54067.1	45	165	3.6	**MLC_3390**	*pepQ*	Subfamily M24B unassigned peptidases
CBW54074.1	0	0		MLC_3460		Subfamily C1A unassigned peptidases
CBW54082.1	0	2		MLC_3540	*lip1*	Family S33 unassigned peptidases
CBW54162.1	49	16	0.3	**MLC_4340**	*lon*	Lon-A peptidase
CBW54168.1	5	16	2.7	**MLC_4400**		Family C56 nonpeptidase homologues
CBW54169.1	0	0		MLC_4410	*abc*	Family C39 unassigned peptidases
CBW54212.1	40	18	0.5	**MLC_4840**	*pepO*	Family M13 unassigned peptidases
CBW54233.1	50	29	0.6	**MLC_5050**	*lip2*	Family S33 unassigned peptidases
CBW54234.1	15	0	0.0	**MLC_5060**	*lip2*	Family S33 unassigned peptidases
CBW54235.1	14	16	1.1	**MLC_5070**	*lip3*	Family S33 unassigned peptidases
CBW54242.1	12	3	0.2	**MLC_5140**	*nagA*	Family M38 nonpeptidase homologues
CBW54257.1	0	0		MLC_5290		Subfamily C1A unassigned peptidases
CBW54260.1	35	66	1.8	**MLC_5320**	*pepV*	Peptidase V
CBW54267.1	0	0		MLC_5390		Family C108 unassigned peptidases
CBW54280.1	0	0		MLC_5520	*lspA*	Family A8 nonpeptidase homologues
CBW54282.1	1	0		MLC_5540	*pepD*	Subfamily S9C unassigned peptidases
CBW54326.1	2	0		MLC_5980		Subfamily S8A unassigned peptidases
CBW54404.1	6	21	2.9	**MLC_6750**	*map*	Subfamily M24A unassigned peptidases
CBW54445.1	0	0		MLC_7150		Family M79 unassigned peptidases
CBW54490.1	7	0	0.0	**MLC_7600**	*pldB*	Family S33 unassigned peptidases
CBW54520.1	6	14	1.9	**MLC_7900**		Esterase EstB
CBW54631.1	0	0		MLC_9010		Subfamily M23B nonpeptidase homologues
CBW54632.1	0	1		MLC_9020		Subfamily M23B nonpeptidase homologues
CBW54642.1	3	0	0.0	MLC_9120		Subfamily S8A unassigned peptidases
CBW53777.1	22	29	1.3	**MLC_0490**		Putative peptidase DUF31
CBW53798.1	3	0	0.0	MLC_0700		O-Sialoglycoprotein endopeptidase
CBW53816.1	0	0		MLC_0880	*pepQ*	Proline dipeptidase
CBW53878.1	0	0		MLC_1500		Papain-like cysteine peptidase superfamily
CBW53944.1	2	1		MLC_2160		Peptidase_M78
CBW53958.1	3	3	0.8	MLC_2300		Inactive homologue of metal-dependent proteases
CBW53994.1	**1**	**10**	**5.8**	**MLC_2660**		**Putative peptidase DUF31**
CBW54029.1	0	0		MLC_3010		Peptidase_M78
CBW54081.1	**1**	**8**	**4.8**	**MLC_3530**		**Papain-like cysteine peptidase superfamily**
CBW54164.1	1	1		MLC_4360		Papain-like cysteine peptidase superfamily
CBW54165.1	12	12	0.9	**MLC_4370**		Papain-like cysteine peptidase superfamily
CBW54170.1	1	0		MLC_4420		Metalloprotease catalytic domain superfamily, predicted
CBW54304.1	30	10	0.3	**MLC_5760**	*clpB*	ATP-dependent Clp protease ATP binding subunit
CBW54318.1	13	11	0.8	**MLC_5900**		Putative peptidase DUF31; *Mycoplasma* IgG protease
CBW54320.1	18	44	2.4	**MLC_5920**		Putative peptidase DUF31; *Mycoplasma* IgG protease
CBW54322.1	4	0	0.0	MLC_5940		Putative peptidase DUF31; *Mycoplasma* IgG protease
CBW54324.1	7	14	1.8	**MLC_5960**		Putative peptidase DUF31; *Mycoplasma* IgG protease
CBW54539.1	1	1		MLC_8090		Putative peptidase DUF31
CBW54606.1	0	0		MLC_8760		Zinc metalloprotease

[a]Mnemonics of genes which were found to be consistently expressed in either the pellet or the supernatant (proportion, >0.001) are indicated with boldface. Proteases whose proportion was significantly higher in the supernatant are indicated with boldface and underlining.

MEROPS database, while 19 additional genes were identified through MaGe data mining. In the closely related subspecies *M. mycoides* subsp. *mycoides*, this number was reduced to 36, as orthologues were either absent or present in the form of pseudogenes (see Table S1B in the supplemental material).

Similarly, the number of protease-coding genes was drastically reduced for *M. capricolum* subsp. *capripneumoniae* ($n = 35$) compared to *M. capricolum* subsp. *capricolum* ($n = 44$) (Table S1A). Only 39 putative protease genes were predicted in the *M. bovirhinis* genome (Table S1C). Thus far, our experimental approach was based on phenotypic detection of protease activity, more specifically, of caseinolytic activity. For the sake of completeness, we also developed a general proteomic approach consisting of the characterization of all secreted proteases detected in Opti-MEM supernatants. For this purpose, mycoplasma cell suspensions were incubated as described above in Opti-MEM for 16 h before being harvested as two separated fractions, i.e., the cell pellet and the supernatant. Both fractions were subjected to MS-MS analysis. Of the 55 putative proteases from *M. mycoides* subsp. *capri* 95010 that were identified after genome mining, 35 were detected by tandem mass spectrometry analysis either in the pellet or in the supernatant (Table 3), whereas 18 of the 39 predicted were detected in *M. bovirhinis* MV5 samples (Table S1C).

FtsH, which is a membrane-bound, cytoplasm-oriented, energy-dependent AAA-positive (AAA^+) protease (20), was used as a control. This protease is a universally conserved protein with well-established localization in the cytoplasm, presenting two hydrophobic domains anchoring it to the membrane. Its ratio within the pellet samples was quite stable, whichever mycoplasma strain was studied, with a mean of 6.7×10^{-3} ($n = 15$; minimum $= 4.5 \times 10^{-3}$; maximum $= 8.5 \times 10^{-3}$) and a supernatant/pellet fold change level which ranged from 0.04 to 0.7, an indication that FtsH was detected mostly in the cell pellet. This preliminary analysis confirmed that our procedure for sample preparation did not induce a noticeable level of release of membrane fragments in the supernatant attributable due to cell lysis or vesicle formation (21). In contrast, a number of proteases were highly expressed and detected both in the pellet and in the supernatant (0.5-fold to 2.5-fold change). This was notably the case for endopeptidases such as PepA (1.2 to 2.9), PepF (0.6 to 2.1), and PepV (1.8 to 3.3).

Proteases located preferentially in the supernatant. To detect which proteases were significantly overrepresented in the supernatant, a supernatant-versus-pellet fold change distribution analysis was performed for each of the strains. This allowed detection of which fold change values could be considered "outlier" values compared to the "normal curve" values of the distribution (see Fig. S1 in the supplemental material). Most supernatant/pellet fold change values were located within the first two intervals (0 to 0.5 and 0.5 to 1), which corresponded to proteins detected mostly in the pellet sample. For values above 1, the curve had a negative exponential shape up to a fold change value of 5, which was considered the outlier limit for that example. The curves for all other strains had similar shapes, but the upper limits differed slightly (data not shown). This analysis allowed the detection of proteases that were preferentially overrepresented in supernatant samples from *M. mycoides* subsp. *capri* 95010 (Table 3) as well as the other members of the *Mycoplasma mycoides* cluster and *M. bovirhinis* (Table S1C).

The most striking one was a serine protease (GenBank accession no. CBW53985.1 and its orthologues, namely, MLC_2570, MCAP_0240, and MCCP01_0297), which was detected almost exclusively in the supernatants (fold change, 397) and not in the pellets from all species of *Mycoplasma mycoides* cluster strains, with the exception of *M. mycoides* subsp. *mycoides*, in which it was not detected. Another one (CBW54056.1; MLC_3280), a predicted lipoprotein with a S41 protease domain, was also detected in 4 of 6 *Mycoplasma mycoides* cluster strains, but with lower fold change values (2 to 13). Various other proteases were overrepresented in the supernatant of *Mycoplasma mycoides* cluster strains. For *M. capricolum* subsp. *capricolum* type strain California kid (Ck), two S41 proteases were detected, namely, MCAP_0240, which is orthologous to *M. mycoides* subsp. *capri* MLC_2570, and MCAP_0329, which is orthologous to CBW54056.1 (MLC_3280) *Mycoplasma mycoides* subsp. *capri* strain 95010. The latter was equally extensively detected in the *M. capricolum* subsp. *capricolum* Ck mutant, suggesting that MCAP_0329 is not involved in the extracellular caseinolytic activity of *M.*

capricolum subsp. *capricolum*. The four proteases that were overrepresented in the supernatant of *M. bovirhinis* strain MV5 displayed a DUF31 domain and were analogues of the *Mycoplasma* IgG protease (MIP) evidenced in *M. mycoides* subsp. *capri* (14). These proteases were also detected in the *M. mycoides* subsp. *capri* 95010 strain, both in the pellet and in the supernatant, but they were not found overrepresented in the supernatant of *Mycoplasma mycoides* cluster strains.

These results fully confirm the data obtained by phenotypic detection, which resulted in the identification of the two main proteases, corresponding to genes MLC_2570 and MBVR141_0224, in *M. mycoides* subsp. *capri* and *M. bovirhinis*, respectively. We were not able to detect any overrepresented proteases in *M. mycoides* subsp. *mycoides* strains (Table S1B). Some proteases were indeed detected in the supernatant but with fold change values that could not be considered to represent deviations from the standard distribution. In contrast, all the other subspecies and strains of the *Mycoplasma mycoides* cluster displayed proteases preferentially secreted into the supernatant.

Identification of caseinolytic proteases by zymography. Culture supernatants obtained after incubation in Opti-MEM were concentrated by lyophilization and analyzed by casein zymography (Fig. 2). A single band of digestion representing an estimated molecular weight of 55 kDa was observed for *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae*, while two bands were observed for *M. mycoides* subsp. *capri*, with one also estimated at 55 kDa and the other estimated at 52 kDa. For *M. mycoides* subsp. *mycoides* Rita, one of the zymograms showed a faint band at 55 kDa (data not shown) but the band was not reproducible and thus was not taken into account for further analysis. Tandem mass spectrometry detected specific peptides from orthologues of MLC_2570 peptidase S41 in all the excised bands (Table 4) from strains of the *Mycoplasma mycoides* cluster. For *M. mycoides* subsp. *capri*, MLC_2570 represented the majority of spectral counts for both the 55-kDa and 52-kDa bands, while few peptides corresponding to other proteases were also detected (MLC_1460 and MLC_3270). There was a marked difference between the actual size of the zymography band, 55 kDa, and the predicted molecular weight of MLC_2570, 74.5 kDa, which suggests proteolytic cleavage. The estimated molecular weight of a protein encompassing all amino acids between the detected N-terminal and C-terminal peptides is 53.5 kDa (Fig. 3). This indicates that the actual cleavage sites may be located very close to the peptides detected at the extremities. For *M. capricolum* subsp. *capripneumoniae*, MCCP01_0297 was the only protease detected in the excised band, and few peptides were detected for it. In the case of *M. capricolum* subsp. *capricolum*, the majority of the peptides detected in the sliced 55-kDa band corresponded to another S41 protease, MCAP_0329. However, MCAP_0240, the orthologue of MLC_2570, was also detected. As for *M. bovirhinis* MV5, the two bands of 85 and 80 kDa contained specific peptides corresponding to MBVR141_0224, a putative DUF31 peptidase.

Assessment of the extracellular protease activity of predicted S41 peptidases by analysis of mutant strains. To confirm the activity of MLC_2570 orthologues, two mutant strains with transposon insertions in these orthologues were studied. One was an *M. capricolum* subsp. *capricolum* strain already described (22), with an insertion in MCAP_0240 (Ck-mut), and the other an *M. mycoides* subsp. *mycoides* mutant previously obtained at CIRAD, with an insertion in MSC_0281 (Rita-mut). These insertions had a drastic effect on caseinolytic activities. Grown in rich m-Hayflick medium, activity of mutant strains was negligible or nonexistent in culture supernatants, but it was still detectable in the cell pellets (Table 2). When incubated in a defined medium, Ck-mut lost its caseinolytic activity, both in the pellet and in the supernatant, while the parental strain displayed an increased level of activity, mostly in the supernatant. The results obtained with Rita-mut were less marked, as the parental strain also failed to show caseinolytic activity after incubation in Opti-MEM. Concordant results were obtained on milk agar plates, where *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp.

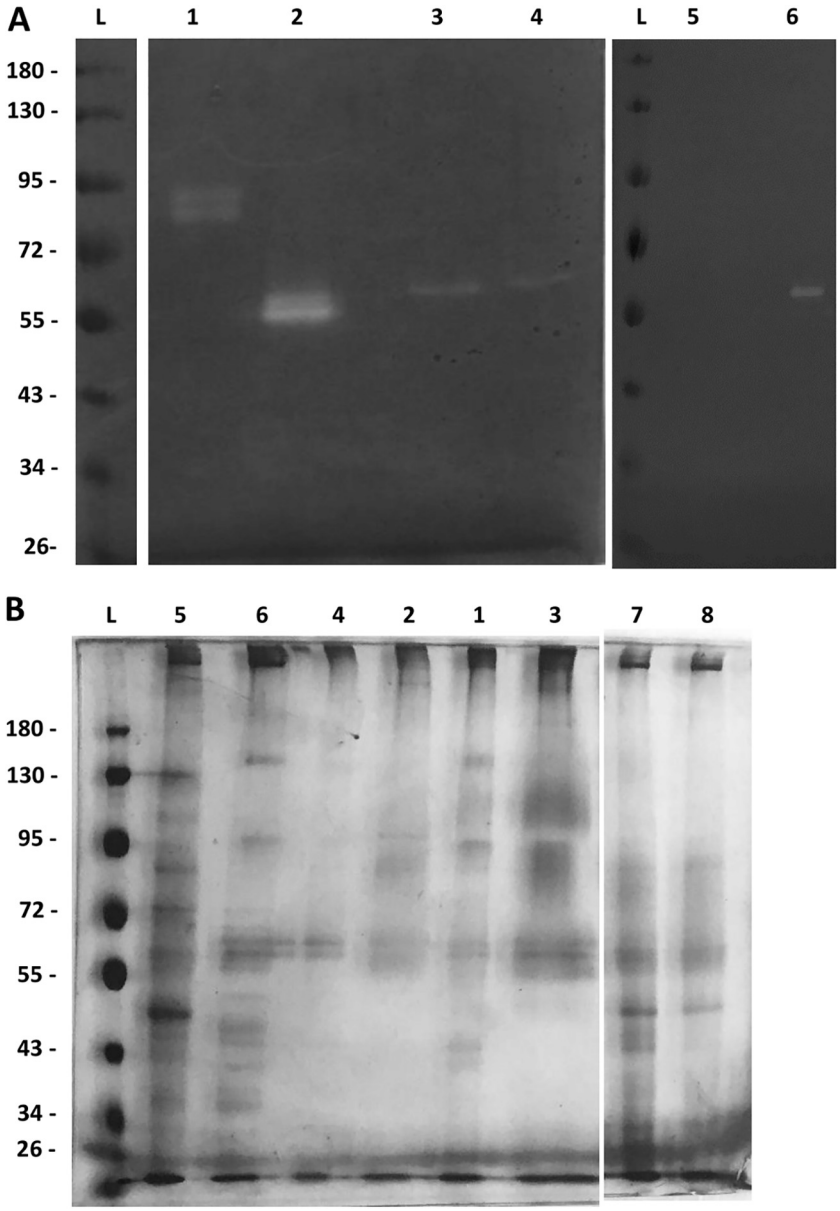

FIG 2 Detection of caseinolytic activity by zymography. Supernatant from mycoplasmas was incubated in supplemented Opti-MEM. (A) Casein zymogram. (B) SDS-PAGE. Lanes L, Ladder PageRuler (top to bottom, 180, 130, 100, 70, 55, 40, 35, and 25 kDa); lane 1, *M. bovirhinis* MV5; lanes 2, *M. mycoides* subsp. *capri* 95010; lanes 3, *M. capricolum* subsp. *capripneumoniae* Abomsa; lane, 4, *M. capricolum* subsp. *capricolum* 94157; lanes 5, *M. capricolum* subsp. *capricolum* Ck-mut; lanes 6, *M. capricolum* subsp. *capricolum* Ck; lane 7, *M. mycoides* subsp. *mycoides* Rita; lane 8, *M. mycoides* subsp. *mycoides* Rita-mut. The discolored bands, showing caseinolytic activity, were cut and then analyzed by tandem mass spectrometry. The zymography picture (panel A) has been spliced to remove the lanes with samples which did not yield any digested bands (*M. arginini*, *M. bovis*, *M. ovipneumoniae*, and *M. mycoides* subsp. *mycoides*).

mycoides mutants completely lost their milk digestion properties, while cultures were clearly visible (Fig. 1). Finally, the digested band of 55 kDa that was observed with *M. capricolum* subsp. *capricolum* Ck in a zymogram performed with the concentrated supernatant was not observed with Ck-mut (Fig. 2). These analyses confirmed the role of secreted S41 peptidase MLC_2570 orthologues in casein degradation. Analysis of concentrated supernatants from two independent replicates of *M. capricolum* subsp. *capricolum* Ck cultures revealed that peptides from 20 predicted surface-exposed proteins, lipoproteins, or proteins bearing N-terminal or C-terminal predicted trans-

TABLE 4 Proteins identified by tandem mass spectrometry from caseinolytic zymogram bands[a]

Strain	Mnemonic	Protein accession no.	Annotation	MW (kDa)	Spectral count			
					55 kDa	52 kDa	85 kDa	80 kDa
M. mycoides subsp. *capri* 95010	**MLC_2570**	**CBW53985.1**	**CHP, predicted TMB protein and tail specific protease**	**74.5**	**35**	**56**		
		CBW54005.1	Pyruvate kinase	53.6	9	5		
		CBW54529.1	ATP synthase alpha chain	58.1	6	2		
	MLC_1460	**CBW53874.1**	Leucyl aminopeptidase	49.8	0	4		
	MLC_3270	**CBW54055.1**	CHP predicted transmembrane protein, peptidase S41	78.3	4	0		
		CBW54480.1	Phosphoglucomutase	63.8	2	0		
M. capricolum subsp. *capricolum* Ckid	MCAP_0329	**ABC01488.1**	lpp, C-terminal processing peptidase family S41	71.7	15			
	MCAP_0240	**ABC01466.1**	**Membrane protein, peptidase S41**	**75.5**	**7**			
		ABC01270.1	Pyruvate kinase	53.6	4			
		ABC01646.1	Membrane protein, putative	206.8	4			
	MCAP_0328	**ABC01790.1**	Membrane protein, peptidase S41	78.9	4			
		ABC01474.1	Arginine deiminase	46.6	3			
		ABC01292.1	ATP synthase F1, alpha subunit	58.0	2			
M. capricolum subsp. *capripneumoniae* Abomsa		CDZ17831.1	lpp, ABC transporter substrate-binding protein	60.1	12			
		CDZ18073.1	Dihydrolipoyllysine-residue acetyltransferase	46.8	10			
		CDZ17832.1	ABC transporter, ATP binding component	59.4	8			
		CDZ17891.1	ATP synthase (subunit alpha, component F1)	58.1	4			
	MCCP01_0297	**CDZ18087.1**	**Putative conserved membrane protein, peptidase S41**	**75.5**	**4**			
M. bovirhinis MV5	MBVR141_0224	**BBA22185.1**	HP, lpp, putative peptidase (DUF31)	104.0			16	70
		BBA22123.1	Hypothetical protein	87.7			68	47
		BBA22285.1	Hypothetical protein	87.2			34	0
		BBA22436.1	Hypothetical protein	98.5			31	0
		BBA22541.1	Surface protein	80.9			26	28
		BBA22061.1	Hypothetical protein	106.0			15	6
		BBA22174.1	Elongation factor G	77.0			11	0
		BBA22209.1	Phosphoketolase	91.5			7	0
	MBVR141_0761	**BBA22491.1**	HP, lpp, putative peptidase (DUF31)	93.9			5	5
	MBVR141_0284	**BBA22211.1**	HP, putative peptidase (DUF31)	88.1			4	0
		BBA22472.1	Membrane protein	90.2			4	1
		BBA22169.1	Hypothetical protein	120.0			2	2

[a]Protein accession numbers with boldface correspond to predicted proteases. Mnemonic designations with boldface and underlining correspond to the deduced gene coding for the caseinolytic protease. CHP, conserved hypothetical protein; DUF, domain of unknown function; HP, hypothetical protein; lpp, lipoprotein; TMB, transmembrane region; MW, molecular weight.

MKLVKKIGFLSLSAISILGPLAMINNLTTDNNLLITKRFLSSSNSNVGLKSYDYINLINN
KYIPAKINLHDHNGIAYIGVKEFLNSLDGLISFSKIKVRPYQNANFYKEKEISYNYKDNK
VVLNSISKYSNNNKTTNYQLEIDSKNKTITVSDNDFFTDIFTFYRRGEEDLNIDFLNTEI
VNKNKHIVFDLNKYGIEILNDQNDLYLPLVLINQLFLNQSNVQLYFNGQSVNLFAYSKTL
GKVELLKQLKHSYLNNQNHIPAGLKDFQYKYLGFLFDHFYGIKLDKNASYKDLFKKYEKY
IKADNTTHYLTSRYLIEQLDDLHSSYLLTGYYNKDLETINKAVLKTTTPRSDRFKDIARR
LSAYYDKELNYKNVYTPDR**KTSVISFKNFEANSAFKIEESLKQAQRDGIKNIVLDVSFNS**
GGYLGTAFEIMGFLTDKPFKSYSYNPLTKEQKVETIKSRFKKYDFNYYVLTSPFSFSAGN
IFPQLVKDNNVAKVIGFKTAGGASAISQAILPTGDIIQLSSNNVLTNKSHQSLEYGVNPD
ITLGFDPFKQTEKFFASAYIQQAINKDTNTLNSIPATHSSVIEPNYVHELVEQPQPLQLS
RKTDETEIKNLNNLFSSIKETERKDAYFVLGALGVVISLAISFVIIKKLLK

FIG 3 Identification of peptides detected by tandem mass spectrometry for the predicted S41 protease (CBW53985.1, MLC_2570) of *M. mycoides* subsp. *capri* strain 95010. The N-terminal and C-terminal transmembrane regions are boxed. The predicted S41 superfamily domain is represented in bold with a boxed serine active site. The specific peptides detected by tandem mass spectrometry in the concentrated supernatant are underlined.

membrane regions were detected at significantly higher levels in the supernatant than in the cell pellet. The results obtained with three Ck-mut cultures showed that the extracellular secretion profile of 17 of these 20 proteins was significantly altered (Table 5). Their extracellular secretion was heavily reduced or even abolished, and 4 of them could no longer be detected either in the supernatant or in the cell pellet. The expression and extracellular secretion levels of 3 of these 20 proteins was unaltered.

***In silico* analysis of serine protease CBW53985.1 (MLC_2570).** Protein CBW53985.1 was by far the most overrepresented protease in the supernatants, especially in *M. mycoides* subsp. *capri* strains. It is 651 amino acids (aa) long and possesses two predicted transmembrane domains located at its N and C extremities (positions 7 to 29 and 627 to 646, respectively) and a predicted signal peptidase I (SPI) site at positions 29 to 30. BLASTP analysis revealed a "C-terminal processing peptidase family S41; peptidase family S41" domain spanning positions 383 to 543 with a high level of probability (2.7 e−23), with an active-site serine at position 477 (Fig. 3). High (>78%) levels of identity were observed with orthologous genes in mycoplasmas of the *Mycoplasma mycoides* cluster (*M. mycoides* subsp. *mycoides*, *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, and *M. leachii*) but also in *M. feriruminatoris* (82.6% identity). Two other proteases of *M. mycoides* subsp. *capri* were detected by BLASTP analysis with lower levels of identity (49.1% and 44.5%, respectively). Both (namely, CBW54055.1 [MLC_3270] and CBW53831.1 [MLC_1030]) had greater sizes (679 aa and 770 aa, respectively) but very similar structures, including an SPI site at the N-terminal extremity and an S41 domain and a transmembrane fragment at the C-terminal extremity. In addition, another *M. mycoides* subsp. *capri* 95010 protein displayed an S41 domain (CBW54056.1; MLC_3280), was predicted to be a lipoprotein, and did not display any C-terminal transmembrane fragment but did yield lower identity values by BLASTP. Similar mycoplasmal S41 serine proteases were detected by performing a BLASTP analysis that excluded the *Mycoplasma mycoides* cluster genomes, notably in *M. agalactiae*, *M. alkalescens*, *M. auris*, *M. bovis*, *M. putrefaciens*, and *M. salivarium* (see the supplemental material). More distantly related S41 bacterial serine proteases were also detected by BLAST analyses of the conserved S41 domain against the nonredundant database (excluding mycoplasmas). Four S41 serine proteases from *Ruminococcus flavefaciens* (WP_009982655.1, WP_028518726.1, WP_082325677.1, and WP_080693401.1) were detected and had very similar features. Interestingly, a typical LPXTG sortase-associated cell wall anchor domain was located shortly before the C-terminal transmembrane fragment in all four S41 *Ruminococcus* peptidases.

DISCUSSION

Proteases are among the largest families of metabolic enzymes. They operate by a variety of mechanisms and are vital to many aspects of bacterial cell life and patho-

TABLE 5 List of proteins whose extracellular secretion was modified in the Ck-mut strain compared to the original Ck strain[a]

Category	Protein accession no.	Spectral count for strain (titer [log/ml]): Ck (9.4) P	Ck (9.4) S	Ck1 (9.7) P	Ck1 (9.7) S	Ck-mut1 (9.1) P	Ck-mut1 (9.1) S	Ck-mut5 (8.9) P	Ck-mut5 (8.9) S	Ck-mut50 (8.7) P	Ck-mut50 (8.7) S	Mnemonic	Location	Annotation
Gene translation abolished	ABC01099.1	1	47	3	72	0	1	0	1	0	0	MCAP_0843	lpp	Transglutaminase
	ABC01278.1	0	44	0	70	0	0	0	0	0	0	MCAP_0860	1 TMB	Hypothetical protein
	ABC01319.1	0	25	0	8	0	0	0	0	0	0	MCAP_0863	—[b]	DUF2570
	ABC01807.1	0	100	11	146	0	0	0	0	0	0	MCAP_0864	1 TMB	Topoisomerase?
Gene translation and protein exosecretion reduced	ABC01143.1	0	50	0	47	4	8	3	4	5	0	MCAP_0351	1 TMB	IgG-blocking virulence domain
	ABC01224.1	0	56	0	38	3	19	3	9	1	4	MCAP_0513	lpp	Transglutaminase-like superfamily
	ABC01376.1	0	157	2	271	0	18	0	9	0	0	MCAP_0862	1 TMB	DUF342
	ABC01488.1	0	43	0	35	0	7	0	3	0	0	MCAP_0329	1 TMB	Peptidase S41
	ABC01574.1	8	161	9	209	0	6	0	0	0	0	MCAP_0861	1 TMB	DUF342
	ABC01698.1	4	106	10	80	30	43	19	18	17	5	MCAP_0514	lpp?	Transglutaminase-like superfamily
	ABC01774.1	0	69	3	57	7	8	5	3	3	0	MCAP_0349	1 TMB	IgG-blocking virulence domain
	ABC01864.1	0	16	0	11	4	4	1	0	0	0	MCAP_0345	1 TMB	IgG-blocking virulence domain
Protein exosecretion reduced or abolished	ABC01302.1	34	187	20	118	81	36	109	29	172	6	MCAP_0115	1 TMB	RecF/RecN/SMC N-terminal domain
	ABC01413.1	15	75	16	65	32	44	34	17	32	3	MCAP_0720	lpp	lppQ
	ABC01444.1	12	59	13	38	22	11	25	2	20	0	MCAP_0348	lpp	Peptidase
	ABC01466.1	24	156	68	214	54	28	63	29	76	20	MCAP_0240	2 TMB	Peptidase S41
	ABC01836.1	3	26	5	18	23	0	27	0	16	0	MCAP_0019	1 TMB	Secreted thousand residue frequently tandem
Gene translation and protein exosecretion unmodified	ABC01469.1	0	23	0	19	1	22	7	39	4	17	MCAP_0607	lpp	Transglutaminase-like superfamily
	ABC01669.1	0	13	0	15	0	28	0	11	0	3	MCAP_0399	2 TMB	Hypothetical protein
	ABC01832.1	0	16	1	22	3	26	3	11	1	2	MCAP_0401	2 TMB	Hypothetical protein

[a]DUF, domain of unknown function; lpp, lipoprotein; P, pellet; S, supernatant; TMB, transmembrane region.

[b]This protein is predicted to be 273 aa long in the Ck genome. However, orthologous genes in *M. capricolum* subsp. *capricolum* genomes are longer and contain one TMB.

genicity (23), such as proteolysis and digestion, cellular respiration, energy storage, transcription, and response to the environment. Proteolytic activity in mycoplasmas was first observed by Longley using inspissated goat or sheep serum for the growth of an organism causing pleuropneumonia in goats (24). This finding prompted the development of methods to evaluate the proteolytic activities of mycoplasmas (25). Only a few *Mycoplasma* species displayed such activity, including *M. arthritidis*, which digested gelatin (26), *M. bovirhinis*, which digested casein, and *M. mycoides*, which digested gelatin, coagulated serum, and casein (27). *M. mycoides* subsp. *capri* strain 95010 is no exception, as 55 protease-coding genes were predicted in its genome consisting of 962 coding DNA sequences (CDS). This subspecies can be considered a model for the mycoplasmas of the *Mycoplasma mycoides* cluster. It is highly pathogenic and can induce lesions in a variety of organs, but it can also be found as a saprophyte in the ears of normal goats (28, 29). This proves its ability to survive in diverse environments of its caprine host, despite possessing a very small genome of 1.15 megabases.

Within these 55 proteases, one, MLC_2570, appeared to be prominent. This protease was detected solely in the supernatant and not in the cell pellet, and it was shown to be caseinolytic. Orthologs of this gene were detected within the *Mycoplasma mycoides* cluster *sensu stricto* but not in closely related mycoplasmas found in ruminants such as *M. cottewii* or *M. yeatsii* nor in the rest of the members of the *Spiroplasma* group of species found in insects or plants. Accordingly, this gene belongs to the group of genes that were most probably acquired via horizontal gene transfer (HGT) by the species of the *Mycoplasma mycoides* cluster to enable their becoming successful ruminant pathogens (30). The most closely related BLASTP bidirectional best hits outside the *Mycoplasma mycoides* cluster were observed with *M. auris*. Best hits were also detected with *M. alkalescens* and other ruminant pathogens such as *M. agalactiae* and *M. bovis*. In such cases, the reciprocal best hits were different protease genes (MLC_1030 and MLC_3270), which seemed to indicate that the HGT involved a number of protease genes, highlighting the importance of these enzymes for evolutionary convergence toward ruminant colonization within divergent mycoplasma lineages. The origin of the HGT may be hinted at by examination of the best hits outside mycoplasma genomes, which corresponded to S41 peptidases of *Ruminococcus* species (WP_093044261.1). They share the global architecture of MLC_2570, with a similar length, an N-terminal SPI domain, an S41 family motif, and a C-terminal transmembrane domain. These *Ruminococcus* proteases possess a typical sortase LPXTG motif shortly upstream from the C-terminal transmembrane domain. These sortase motifs are typical of the Gram-positive proteins that are expressed at the cell surface through a covalent linkage to their cell wall (31, 32). It is tempting to draw a parallel with a possible secretion mechanism occurring in mycoplasmas of the *Mycoplasma mycoides* cluster. However, there are still some clues missing to explain the extracellular secretion of the protease. Neither a signal peptidase I nor a sortase-coding gene has been identified in the *Mycoplasma mycoides* cluster genomes (33). An alternative cleavage process, based on endoproteolysis, may be involved here. Analyzing an *M. capricolum* subsp. *capricolum* Ck mutant lacking the MLC_2570 orthologue, it was not only the S41 protease whose exosecretion in the spent medium was altered but also most of the other proteins that were seen to be overrepresented in the supernatant of the parental Ck strain.

These results suggest that this S41 protease could be involved in the posttranslational processing of many mycoplasma surface-exposed proteins. Further work is needed to determine the cleavage site of the protease and verify which surface proteins are candidate substrates. In mycoplasmas, the proteolysis of surface-exposed proteins seems to be common. This is the case for the MALP-404 lipoprotein of *M. fermentans* (34), with the release of a soluble lipoprotein fragment and the alteration of the surface phenotype leading to a shorter membrane-anchored fragment acting as a Toll-like receptor macrophage-activating lipopeptide. This study had clearly demonstrated that the release of the MALP-404 fragment entailed extracellular processing, considered a new mechanism for the "secretion" of hydrophilic proteins in mycoplas-

mas. This mechanism is widely distributed in mycoplasmas, and it has been extensively studied in *M. hyopneumoniae* (35), where endoproteases are responsible for ectodomain shedding (36), with notable consequences with respect to adhesion and plasminogen activation. The posttranslational processing of adhesion is not limited to mycoplasmas found in animals, as it is also present in the plant pathogen *Spiroplasma citri*, where it affects adhesion-related proteins (*S. citri* ARP [ScARP]) (11).

Studying medium-secreted proteins is difficult in mycoplasmas, which need complex and rich media for their growth. This is the reason why we adopted the same strategy that allowed us to characterize secreted polysaccharides (37). Washed mycoplasma cells were incubated into a defined medium, Opti-MEM, which contains only a very limited amount of proteins consisting of growth factors such as insulin and transferrin. This should allow the mycoplasma to maintain some metabolic activities while obviously not enabling its multiplication. This strategy proved efficient, as medium-exported proteins were clearly detected and could be identified by tandem mass spectrometry. However, since this medium did not allow the multiplication of the mycoplasma, its metabolism may have been modified by this stress through a classical "stringent response," as seen in other bacteria (38). The RelA/SpoT homologue superfamily, which is involved in the regulation of (p)ppGpp alarmone during stress, may not be present in some mycoplasma species (39). However, it is present in the *Mycoplasma mycoides* cluster and specific spectral counts of RelA were detected in the mycoplasma pellets of all studied strains. In the case of *M. mycoides* subsp. *capri*, the secretion of S41 peptidase in the medium is certainly not specific to the incubation into a defined medium, as a high level of caseinolytic activity was also detected in the highly enriched m-Hayflick spent medium. For some species, such as *M. mycoides* subsp. *mycoides*, and, to a lesser extent, *M. capricolum* subsp. *capripneumoniae*, the caseinolytic activity was more pronounced in rich medium than in Opti-MEM medium.

This raises the issue of the ability of the various mycoplasmas to adapt to stress, a field which is emerging now for these organisms (40, 41). A lower level of adaptability for *M. mycoides* subsp. *mycoides* and *M. capricolum* subsp. *capripneumoniae* would not be surprising, as these species possess degenerate genomes compared to the close relatives from which they emerged (42, 43). Their reduced gene repertoire may be associated with a restricted ecological niche, i.e., the ruminant lungs, and with an inability to cope with stress encountered in other body compartments. The genes coding for the S41 peptidases are still functional in *M. capricolum* subsp. *capripneumoniae*, despite its degenerated genome. This was very noticeable on milk agar, where casein digestion was clearly visible in spite of colony growth being barely noticeable. Expressing and exporting a protease must therefore bring a fitness advantage to these fastidious bacteria. As mycoplasmas are dependent on the supply of peptides for their metabolism, protein digestion by S41 protease could be a first step before further degradations by peptidases (Pep A-F-O-Q-V) and uptake by the oligopeptide ABC transporters. The *in vivo* relevance of these events, notably in terms of virulence, has yet to be evaluated. The release of peptides and active proteases could well represent major events in the pathological process by disrupting the delicate environment of the lung alveoli. Concomitantly, the antigenic variation that it involves on the cell surface may have profound effects on the interactions with the host innate and adaptive immune responses.

MATERIALS AND METHODS

Mycoplasma strains and culture conditions. At least 2 strains from each of 13 mollicutes (sub)species that can be isolated from ruminant lungs were analyzed in this study (Table 1). The MSC_0281::pMT85/2res transposon mutant of *M. mycoides* subsp. *mycoides* strain 8740 Rita (Rita-mut), with an insertion in the orthologue of *M. mycoides* subsp. *mycoides* PG1^T MSC_0281, was selected from a mutant bank produced at CIRAD. The procedures for transformation and identification of pMT85/2res transposon insertion site sequences have been previously described (44). The *ctpA*::Tn*4001t* mutant of the California Kid type strain, or Ck^T (Ck-mut), was kindly provided by M. Brown (Department of Infectious Diseases and Pathology, College of Veterinary Medicine, University of Florida). This mutant was generated by random mutagenesis, with the Tn*4001t* transposon being inserted in the *ctpA* (MCAP_0240) gene (22). All other strains were cultured in either M-Hayflick medium (44) or commercialized pleuropneumonia-like organ-

ism (PPLO) modified broth (Indicia, France). Opti-MEM GlutaMAX medium (Gibco), depleted of proteins except for minute amounts of insulin and transferrin, was used to characterize the extracellular proteases. This defined medium was supplemented with 0.4% (wt/vol) pyruvic acid, 0.02% (wt/vol) DNA (herring sperm) (to maintain cell viability), and 0.1% (wt/vol) ampicillin, and the reaction mixture was subjected to sterilization by passage through a 0.1-μm-pore-size filter unit (Millex low protein binding Durapore, polyvinylidene difluoride [PVDF]). Mycoplasma cultures were incubated at 37°C and 5% CO_2 in a humid atmosphere. Culture titers were evaluated by performing six 10-fold dilutions in m-Hayflick and plating 10-μl aliquots of the last three dilutions on agar plates to quantify viable bacteria by colony counting.

Determination of global caseinolytic activity. The proteolytic activity of mycoplasmas was investigated using casein as the substrate. The overall caseinolytic activity of the mycoplasma strains was assessed by measuring the degradation of fluorescent casein using a protease detection kit (Jena Bioscience PP404S). The microtiter plate operation protocol was used following the manufacturer's instructions, and fluorescence was measured using an Enspire 2300 fluorimeter (Perkin Elmer), with excitation at 490 nm, emission at 525 nm, 100 flashes, and a top measurement height of 9.5 mm. With the exception of Fig. S2, where the original relative fluorescence units (RFU) were retained, each fluorescence measurement was normalized using the kit's positive control and the corresponding medium negative control, according to the following formula (with relative activity [RA] expressed as a percentage): RA = 100 × {[(log RFU sample) − (log RFU medium)]/[(log RFU kit_positive_control) − (log RFU medium)]}.

This approach was validated using strains belonging to three different *Mycoplasma* species, namely, *M. mycoides* subsp. *capri* (*M. mycoides* subsp. *capri*) strain 95010, *M. mycoides* subsp. *mycoides* strain Gladysdale, and *M. bovis* strain L2. The caseinolytic activity was measured in serial 10-fold dilutions, which were then incubated. The last tube showing some turbidity was considered to be in the exponential phase of growth, while the preceding tubes were at later stages of growth, up to the stationary phase within the first tube of the series. The fluorescence was shown to be stable and at its maximum in the stationary phase for all three species (Fig. S2A). Under our experimental conditions, the choice of medium used for protease detection (Indicia versus m-Hayflick) had no significant impact on the overall activity of *M. mycoides* subsp. *capri* 95010, although m-Hayflick yielded a lower background level (Fig. S2B). Finally, the robustness of this assay was demonstrated by analyzing three independent cultures of the same strains, which provided reproducible results (Fig. S2C).

The fluorescent casein test was also used to evaluate whether the activity was present in the culture supernatant or associated with the mycoplasma pellet. For this purpose, 1-ml volumes of stationary-phase cultures were centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatants were filtered through a 0.1-μm-pore-size filter unit (Millex low protein binding Durapore, PVDF), while the pellet was resuspended in the original culture volume (i.e., 1 ml) of phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM NaCl, pH 7.8). The absence of residual viable mycoplasmas was confirmed by plating 10-μl volumes of the filtered supernatant onto agar medium. The caseinolytic activity was measured as described above for the two fractions. The same method was applied for the determination of global caseinolytic activity in exoproteome extracts. For this purpose, mycoplasmas grown in m-Hayflick until the late exponential phase of growth were centrifuged 20 min at 12,000 × *g*, washed twice in non-supplemented Opti-MEM medium, and concentrated thrice in supplemented Opti-MEM. After incubation for 16 h in supplemented Opti-MEM, the cultures were centrifuged at 12,000 × *g* for 20 min at 4°C. The pellets were resuspended in the same volume of PBS, and the supernatants were filtered through 0.1-μm-pore-size filter units. The caseinolytic activities of the 16-h cultures incubated in Opti-MEM, as well as the supernatant and pellet corresponding to each culture, were determined as indicated above. Viability losses during Opti-MEM incubation were evaluated by titration of the washed culture transferred into Opti-MEM medium (time zero [T0] titer) and of the same culture after the 16-h incubation period.

Assessment of proteolytic activity on milk agar. Milk agar plates were prepared by adding 0.4% (wt/vol) dried skimmed milk powder to m-Hayflick agar medium. Mycoplasma stationary-phase cultures (7 μl) were plated and incubated for 60 h. Casein degradation was then assessed against a dark background by evaluating the presence of a translucent area around the culture, indicating casein degradation (−, no translucent area; +, blurred and small translucent area; ++, sharp and large translucent area).

Identification of *M. mycoides* subsp. *capri* 95010 and *M. bovirhinis* MV5 genes coding for putative protease motifs. The table listing the proteases from *Mycoplasma mycoides* was retrieved from the MEROPS peptidase database (https://www.ebi.ac.uk/merops/cgi-bin/speccards?sp=sp003189;type=peptidase). As this list included peptidases that were identified in three subspecies or serotypes, namely, *M. mycoides* subsp. *mycoides*, *M. mycoides* subsp. *capri* serotype LC, and *M. mycoides* subsp. *capri* serotype capri (locus tags MSC, MLC, and MMCAP1, respectively), the corresponding genes in the genome of *M. mycoides* subsp. *capri* LC strain 95010 (NC_015431) were identified and duplicates discarded. This resulted in a table with 36 entries. However, additional genes bearing putative protease motifs could also be identified in genome annotations. To retrieve them, a query on the MaGe website was performed using the "search by keywords" tool and using "Protease OR peptidase" as the query (https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php). This led to the identification of 19 additional genes potentially coding for proteins with peptidase activity. In addition, the orthologous genes found in the *Mycoplasma mycoides* cluster species were identified using the MaGe interface. As *M. bovirhinis* genomes were not integrated in the MEROPS database, any proteins that may be potential peptidases were retrieved by searching using "peptidase OR protease" in the genome of strain HAZ 141_2 (accession AP018135).

Tandem mass spectrometry analysis of the exoproteome. Mycoplasma cultures, incubated 16 h in supplemented Opti-MEM as described above, were centrifuged at 12,000 × *g* for 20 min at 4°C. The cell pellets were resuspended in PBS and standardized to obtain 1 mg/ml of proteins, and the culture supernatants were filtered through 0.1-μm-pore-size filters as described above. The Opti-MEM filtered supernatants were freeze-dried (2.5 ml per vial). The freeze-dried supernatants were first reconstituted with 120 μl of sterile MilliQ water and then supplemented with 120 μl of 2× Laemmli buffer. Samples were incubated for 5 min at 99°C and then subjected to SDS-PAGE on a 4% to 12% NuPage gel (Invitrogen) with MES (morpholineethanesulfonic acid) buffer (Invitrogen) for a short electrophoretic migration, as described previously (45). The whole-protein content from each well was extracted as a single polyacrylamide band, processed for in-gel digestion, and subjected to proteolysis with trypsin (Roche) using 0.01% ProteaseMAX surfactant (Promega) for 1 h at 50°C. The resulting peptide fractions were analyzed with a Q-Exactive HF tandem mass spectrometer (Thermo) coupled with an UltiMate 3000 liquid chromatography (LC) system (Dionex-LC Packings) and operated in data-dependent mode as previously described (46). Peptides were analyzed along a 90-min gradient of acetonitrile with scan cycles initiated by a full scan of peptide ions in the Orbitrap analyzer, followed by high-energy collisional dissociation and MS/MS scans of the 20 most abundant precursor ions with 2+ or 3+ charges only. Full-scan mass spectra were acquired from *m/z* 350 to 1,800 at a resolution of 60,000. Ion selection for MS/MS fragmentation and measurement was performed by applying dynamic exclusion for 10 s. MS/MS spectra were assigned to peptide sequences by the MASCOT Daemon 2.6.0 search engine (Matrix Science) searching against the corresponding annotated theoretical proteome database (*M. mycoides* subsp. *capri* [GenBank accession no. FQ377874.1], *M. mycoides* subsp. *mycoides* [CP002107.1], *M. capricolum* subsp. *capricolum* [CP000123.1], *M. capricolum* subsp. *capripneumoniae* [LM995445.1], and *M. bovirhinis* [AP018135.1]) with the following parameters: full-trypsin specificity, maximum of two missed cleavages, mass tolerances of 5 ppm on the parent ion and 0.02 Da on the MS/MS, carboxyamidomethylated cysteine (+57.0215) as a fixed modification, and oxidized methionine (+15.9949) and deamidation of asparagine and glutamine (+0.9848) as variable modifications. Only peptide matches presenting a MASCOT peptide score with a *P* value of less than 0.05 were retained. Proteins were considered identified when at least two peptides were assigned. For each protein, peptide-to-spectrum assignments were summed (spectral counts) and compared per conditions.

Method for identification of putative proteases enriched in supernatants versus cell pellets. The number of detected spectral counts was recorded for each of the putative proteases in both fractions. As the total amount of spectral counts could differ from one sample to another, ratios were calculated by dividing the detected spectral count for each protein by the total number of spectral counts detected in the sample. This ratio yielded estimated proportions of the protein in the samples that could be used for comparisons of one sample to another. A secondary ratio, or supernatant/pellet fold change value, was then calculated by dividing the ratio obtained for one protein in the supernatant by that obtained in the pellet. The distribution of fold change values was evaluated by performing a frequency curve analysis (Microsoft Excel), with a range of 0 to 33 and intervals of 0.5 to detect outlier values.

Casein zymography. To identify which proteases were able to digest casein, concentrated supernatants and mycoplasma cell pellets were first analyzed by SDS-PAGE (7.5% acrylamide) followed by silver staining. Zymogram preparations were performed on 160-by-180-mm, 1-mm-thick gels. Running gels contained 7.5% (wt/vol) acrylamide–1.5 M Tris (pH 8.8) buffer–0.1% (wt/vol) SDS–ammonium persulfate, supplemented with 0.1% (wt/vol) casein and tetramethylethylenediamine (TEMED) before polymerization. Stacking gels contained a mixture of 3.75% (wt/vol) acrylamide, 0.5 M Tris (pH 6.8), 0.1% (wt/vol) SDS, and ammonium persulfate, supplemented with TEMED before polymerization. A 10-μg volume of each sample was loaded into each well of SDS-PAGE and zymogram gels, avoiding a boiling step. The migration conditions were 70 V (constant) through the stacking gel and 25 mA (constant) through the running gel (all at 4°C) and were maintained until the migration front reached the bottom of the gel. The gel was washed twice for 30 min in washing buffer (2.5% [vol/vol] Triton X-100, 50 mM Tris-HCl [pH 7.5], 5 mM $CaCl_2$, 1 μM $ZnCl_2$) and incubated for 24 h in an incubation buffer that was similar to washing buffer except that the concentration of Triton X-100 was 1% instead of 2.5% (vol/vol). The gel was then stained with Bio-Safe staining solution (Bio-Rad) following the manufacturer's instructions. Active casein proteases, revealed as translucent bands, were collected for further identification analysis by mass spectrometry. The cell pellets and concentrated supernatants were prepared in a similar way for mass spectrometry analysis.

Analysis of serine protease CBW53985.1 (MLC_2570). The search for signal peptidase motifs was performed online with the SignalP v5.0 server for Gram-positive bacteria (http://www.cbs.dtu.dk/services/SignalP/), while transmembrane regions were detected using the TMHMM server, v2.0 (http://www.cbs.dtu.dk/services/TMHMM/). BLASTP analysis was performed using the whole protein CBW53985.1 as the query through the Genoscope MaGe interface with available genomes and then through the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the conserved S41 region of CBW53985.1 on the nonredundant database and excluding mycoplasmas (*Mycoplasmatales*; TaxID: 2085).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01439-19.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

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