INFECTION AND IMMUNITY, Sept. 1994, p. 3793–3800
0019-9567/94/$04.00+0

Vol. 62, No. 9

A 48-Kilodalton *Mycoplasma fermentans* Membrane Protein Induces Cytokine Secretion by Human Monocytes

DAVID A. KOSTYAL,[1]* GARY H. BUTLER,[2] AND DONALD H. BEEZHOLD[1]

Laboratory of Macrophage Biology, Guthrie Foundation for Medical Research, Sayre, Pennsylvania 18840,[1] and Department of Microbiology, Coriell Institute for Medical Research, Camden, New Jersey 08103[2]

Received 18 January 1994/Returned for modification 29 March 1994/Accepted 22 June 1994

***Mycoplasma fermentans* is one of several *Mycoplasma* species that have been reported to stimulate tumor necrosis factor (TNF) secretion from monocytes. This activity has been associated primarily with the mycoplasma membrane fraction. In this article, we have characterized a membrane protein that stimulates TNF and interleukin 1β secretion. The TNF-releasing activity partitioned into the Triton X-114 detergent phase, suggesting that the molecule is hydrophobic. The secretion of TNF is elevated in the presence of serum, which suggests that a serum component may play a role in the interaction between this mycoplasma protein and monocytes. Treatment of monocytes with monoclonal anti-CD14 antibody had no effect on the levels of TNF-releasing activity. By using the monocyte Western blot (immunoblot) technique, we have determined the molecular mass of the active molecule to be 48 kDa. This molecule appears to be distinct from the recently described family of variable lipoproteins of *M. fermentans*. Mycoplasma particulate material treated with proteinase K lost all inducing activity, whereas lipoprotein lipase-treated samples retained some level of activity.**

Mycoplasma fermentans is a member of the class *Mollicutes*, which consists of gram-positive wall-less procaryotes. Members of *Mollicutes* are important pathogens in animals and in humans are a cause of atypical pneumonia (*Mycoplasma pneumoniae*) and nongonococcal urethritis (*Ureaplasma urealyticum*). Recent evidence has implicated members of *Mollicutes* in several infectious etiologies (16). Mycoplasma contamination in tissue culture has been shown to have an antiproliferative effect on certain tumor cell lines. This antiproliferative effect results from the depletion of arginine from the culture medium by mycoplasma arginine deiminase (25). The addition of L-arginine to the culture medium restores the cells to their normal rate of proliferation. Arginine deiminase has also been shown to inhibit tumor growth in vivo (34). Mycoplasmas have also been shown to exert a number of effects on immune cells in culture. Among these are B-cell and T-cell activation and the induction of cytokine secretion by human and mouse monocytes. The B-cell mitogenic activity from *M. arginini* has been associated with proteins of molecular masses of 88, 84, 74, 70, and 61 kDa (29). *Mycoplasma hyorhinis* possesses a B-cell mitogen with a molecular mass of greater than 90 kDa (27). Likewise, a T-cell mitogen from *Mycoplasma arthritidis* has been identified as a protein of 26 kDa (14). This protein belongs to a class of polyclonal T-cell activators known as superantigens (9).

Many species of *Mollicutes* are capable of inducing tumor necrosis factor (TNF) and interleukin 1β (IL-1β) release from mouse and human monocytes (23). Induction of cytokine release by *Spiroplasma* membranes was associated with two proteins, one with a molecular mass of greater than 68 kDa and the other with one of approximately 15 kDa (31). These proteins are thought to be acylated on the basis of their hydrophobic nature. *M. fermentans* has also been shown to induce TNF and IL-1β release from human monocytes. This activity was associated with high-molecular-mass material (10^6 Da) (26) and membrane preparations (11). However, the identification of specific molecules responsible for TNF induction has remained elusive. By using the monocyte Western blot (immunoblot) assay, we have identified a 48-kDa membrane protein that has the ability to induce TNF release from normal human monocytes.

* Corresponding author. Mailing address: Laboratory of Macrophage Biology, Guthrie Foundation for Medical Research, 1 Guthrie Square, Sayre, PA 18840. Phone: (717) 882-4619. Fax: (717) 882-4666.

MATERIALS AND METHODS

Cell culture. *M. fermentans* sb51 (strain incognitus) was originally obtained from S. Lo (Armed Forces Research Institute, Washington, D.C.). Organisms were grown aerobically in BBL PPLO broth containing 10% horse serum, 4% fresh yeast extract, and 100 U of penicillin G per ml for 4 days at 37°C. Mycoplasmas were pelleted at 8,000 × *g*, washed with 250 ml of 0.25 M NaCl–0.02 M Tris (pH 7.2) and then briefly in 30 ml of H_2O, and finally resuspended in 50 ml of H_2O. Cells were sonicated three times with a Branson Sonifier (model 250) with a 3-mm microtip for 10 s at 4°C at a setting of 5. Lysates were then centrifuged at 100,000 × *g* for 2 h. The supernatant, floated lipids, and pellet were collected separately.

L929 cells for TNF bioassay were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 IU of penicillin per ml, and 100 μg of streptomycin per ml.

Reagents. Monoclonal antibodies to CD14 were purchased from Zymed Laboratories (South San Francisco, Calif.). An IL-1β enzyme-linked immunosorbent assay kit was purchased from Endogen (Boston, Mass.), and assays were performed as described in the manufacturer's protocol. Protein concentrations were determined by using the bicinchoninic acid assay (Pierce, Rockford, Ill.).

Triton X-114 phase separation. Mycoplasma lysate material or protein fractions were separated into hydrophilic and hydrophobic fractions by the Triton X-114 phase separation method of Bordier (6). Samples were solubilized in lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5%

Triton X-114 (Sigma Chemical Co., St. Louis, Mo.), and 1.0 mM phenylmethylsulfonyl fluoride. Samples were centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant (450 μl) was layered onto a 300-μl cushion consisting of 6% sucrose, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.06% Triton X-114, incubated for 3 min at 37°C, and then centrifuged at 300 × *g* for 5 min. The aqueous phase was removed and adjusted to 0.5% Triton X-114, chilled to 0°C, placed over the original sucrose cushion, and reextracted. The final aqueous phase was removed, and both aqueous and detergent fractions were brought up to equal volumes in phosphate-buffered saline (PBS). Triton X-114 was removed by precipitating proteins by the addition of 10 volumes of cold acetone. Proteins were recovered by centrifugation at 16,000 × *g* for 10 min at 4°C. Protein pellets were dissolved in PBS, and the protein concentration was determined as described above.

Purification and stimulation of monocytes. Monocytes were isolated from peripheral blood as described previously (15). Briefly, venous blood drawn from healthy volunteers was collected into heparin (10 U/ml), and the peripheral blood mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (1,000 × *g*, 20 min). The peripheral blood mononuclear cells were washed three times with Hanks balanced salt solution and resuspended in RPMI 1640 containing 10% FCS. The peripheral blood mononuclear cells (2×10^6/ml) were plated into 24-well tissue culture plates (Linbro; Flow Laboratories) and allowed to adhere for 2 h at 37°C. Nonadherent cells were removed by washing three times with Hanks balanced salt solution and the adherent cells were cultured in serum-free or 10% FCS–RPMI 1640. The adherent cells were 93 to 95% monocytes as determined by Giemsa staining and phagocytosis of latex beads. The monocytes were stimulated with mycoplasma fractions for 18 h, and supernatants were collected, centrifuged (10,000 × *g*, 2 min), and assayed for TNF activity. Polymyxin B (10 U/ml) was added to all mycoplasma fractions and incubated at room temperature for 30 min before being added to monocyte cultures to neutralize any contaminating endotoxin. In all experiments, controls were incubated in the presence of 10% FCS and in the absence of polymyxin B. In assays involving monoclonal antibodies to monocyte cell surface molecules, monocytes were preincubated with 1 μg of anti-CD14 monoclonal antibodies (Zymed) per ml for 1 h before the addition of sample. Incubations were allowed to proceed for 6 h before supernatants were harvested. TNF alpha (TNF-α) activity was determined by using the L929 assay as described previously (4).

Monocyte Western blot analysis. Samples were mixed with Laemmli reducing sample buffer (17) heated to 55°C before being applied to a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and electrophoresed at 20 mA until the dye front reached the bottom of the gel. Molecular weight markers (Pharmacia, Piscataway, N.J.) were used as standards. The gels were either silver stained (Bio-Rad, Richmond, Calif.) or equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol) for 5 min and then transferred to 0.1-μm-pore-size nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) at 150 mA for 3 h (35). The monocyte Western blot assay was performed by the previously described method (1, 36). In brief, strips (0.5 by 1.0 cm) were cut from the nitrocellulose blot, placed in a tube, and dissolved in 1 ml of dimethyl sulfoxide. Antigen-coated particles were then formed by the dropwise addition of 3 ml of carbonate buffer (pH 9.6), with constant mixing. The precipitate was washed twice with Hanks balanced salt solution and resuspended in 1 ml of serum-free RPMI 1640. Fifty microliters of each sample was used to stimulate the monocytes.

Proteinase K digestion. Twenty-five micrograms of mycoplasma-soluble or particulate fraction protein was adjusted to 50 mM Tris (pH 8.8)–150 mM NaCl. Samples were then incubated with 25 μg of proteinase K (Boehringer Mannheim, Indianapolis, Ind.) per ml at 37°C for 12 h in the presence or absence of 1% SDS (7). Samples were then mixed with SDS sample buffer, separated by SDS–10% polyacrylamide gel electrophoresis (SDS–10% PAGE), blotted to nitrocellulose, and assayed in the monocyte Western blot assay.

Lipoprotein lipase digestion. Twenty-five micrograms of the particulate material was adjusted to 50 mM Tris (pH 7.4)–150 mM NaCl–1 μM phenylmethylsulfonyl fluoride–1 μM leupeptin and incubated overnight at 37°C with 80 U of lipoprotein lipase (Sigma).

RESULTS

Characterization of the TNF-inducing molecule. Mycoplasma cell lysates were fractionated into soluble or cytoplasmic (100,000 × *g* supernatant) and particulate or membrane (100,000 × *g* pellet) fractions and assayed for their ability to induce TNF-α release by using the monocyte Western blot. The solid lines in Fig. 1 show that both fractions contain TNF-α-inducing activity, and this activity was associated with a molecule of an apparent molecular mass of between 41 and 49 kDa. Most of the activity was associated with the particulate fraction since it was more than sixfold higher than that of the soluble fraction.

While mycoplasmas do not contain lipopolysaccharide (LPS) in their membrane, they do contain other molecules, such as lipoglycans, which could be responsible for inducing TNF release. To determine whether the molecule responsible for TNF release was a protein, samples were treated with proteinase K and assayed for TNF-inducing activity. The dashed lines in Fig. 1 show that proteinase K treatment abolished all cytokine-inducing activity, indicating that the molecule was proteinaceous in nature. The photo insert next to each graph shows the protein profile of each fraction before and after enzyme treatment. Proteinase K treatment removed all detectable proteins from both fractions. This coincides with the loss of TNF-inducing activity in these two samples.

Cytokine-inducing activity partitions into the Triton X-114 detergent phase. Triton X-114 phase partitioning was done to determine whether the cytokine-inducing component was hydrophobic. In all experiments, 1 μg of protein was used to stimulate monocytes. Samples were incubated with polymyxin B (10 U) for 30 min before their addition to monocyte cultures to ensure that the induction of TNF release was due to mycoplasma-derived material and not LPS contamination. As shown in Table 1, the TNF- and IL-1β-inducing activity of the lysate material partitions into the detergent phase. The small amount of activity seen in the aqueous phase is within the standard error of the controls. Additionally, Table 1 shows that the cytokine-inducing activity found in both the soluble and particulate fractions also partitions into the detergent phase. This indicates that the cytokine-inducing material is very hydrophobic in nature. The levels of TNF induced by 1 μg of detergent phase protein are equal in both the soluble and particulate fractions. The protein compositions of the two fractions, however, are very different. Table 2 shows that the soluble fraction is composed of approximately 75% aqueous- and 25% detergent-phase proteins. The composition of the particulate fraction is just the opposite, 25% aqueous and 75% detergent. Therefore, the total amount of TNF induced by the particulate fraction in Fig. 1 is much more than that produced by the soluble fraction.

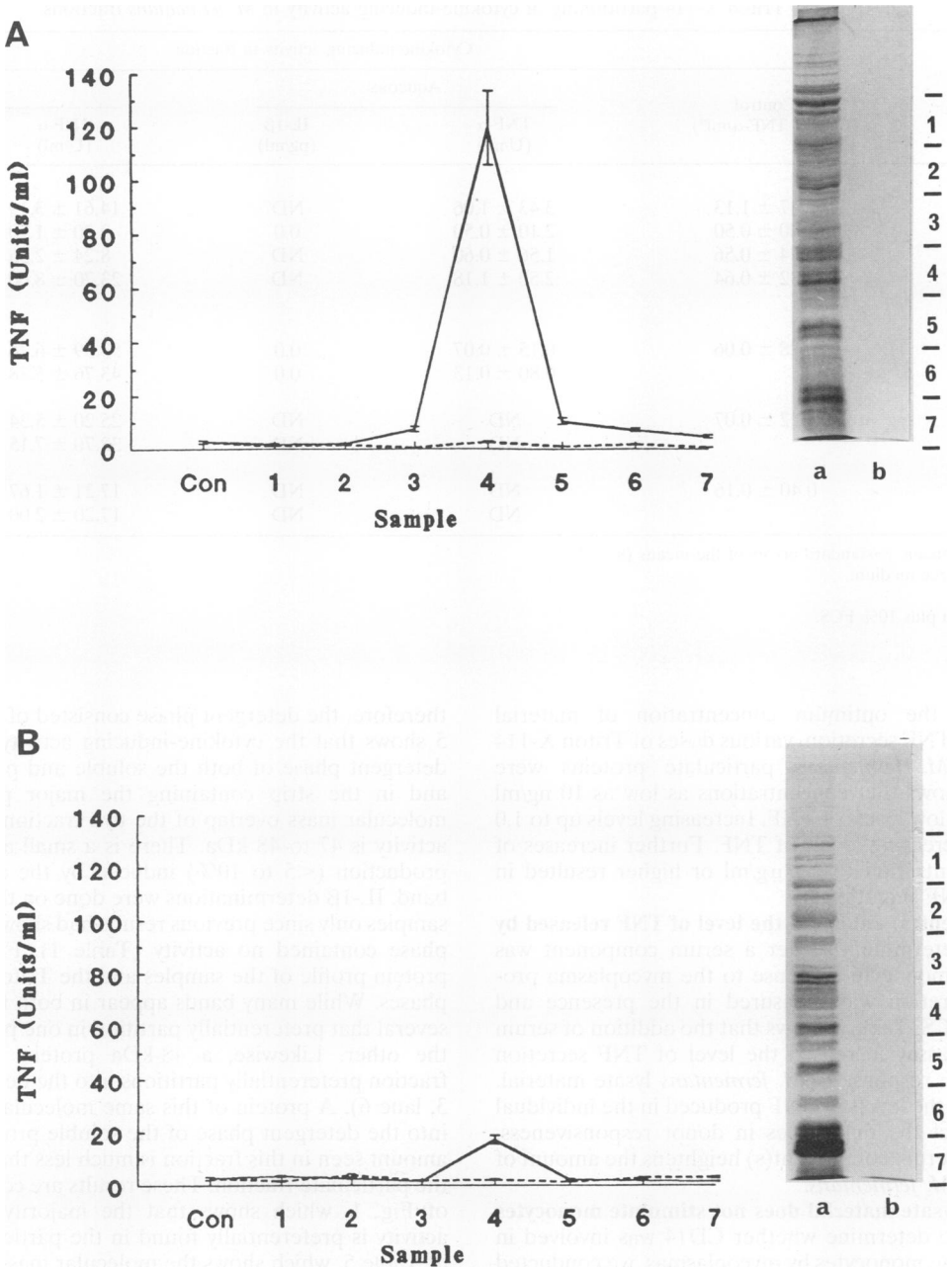

FIG. 1. Proteinase K treatment effects on TNF-inducing activity. *M. fermentans* membrane (A) or cytoplasmic fraction (B) proteins (12.5 μg) were separated by SDS–10% PAGE and blotted onto nitrocellulose. Each lane was cut into 0.5-cm strips and assayed in the monocyte Western blot assay for the induction of TNF release by using normal human monocytes. Solid lines represent untreated samples, while dashed lines represent the same samples treated with proteinase K. The ranges of the molecular masses (in kilodaltons) of the proteins on the nitrocellulose strips are as follows: 1, 100 to 80; 2, 80 to 62; 3, 62 to 49; 4, 49 to 41; 5, 41 to 35; 6, 35 to 31; 7, <31. Values on the graph are given in mean units of TNF ± standard errors of the mean. The photo inserts are silver-stained gels of the samples (lanes: a, untreated; b, proteinase K treated). The graph is representative of three experiments. Con, control.

Mycoplasmas possess many membrane proteins that are modified by the covalent attachment of fatty acids. These acylated proteins have been shown to partition into the Triton X-114 detergent phase (38). The exclusive partitioning of the TNF-inducing activity into the Triton X-114 detergent phase demonstrates its strong hydrophobic nature and suggests that the inducing moiety may be an acylated protein. To test whether a lipid moiety also plays a role in TNF induction, membrane samples were incubated with lipoprotein lipase in the presence of protease inhibitors. Figure 2 shows that the TNF-inducing activity was reduced 40 to 50% by the enzyme treatment but was not totally abolished as it was with the proteinase K treatment. The photo insert shows no detectable difference in the protein profiles of the treated and untreated samples. This suggests that the loss of activity was not due to protein degradation as was seen with proteinase K treatment.

TABLE 1. Triton X-114 partitioning of cytokine-inducing activity in *M. fermentans* fractions

Sample	Cytokine-inducing activity in fraction				
	Control (U of TNF-α/ml[a])	Aqueous		Detergent	
		TNF-α (U/ml)	IL-1β (pg/ml)	TNF-α (U/ml)	IL-1β (pg/ml)
Cell lysate[b]					
Expt 1	2.47 ± 1.13	3.43 ± 1.06	ND[c]	14.61 ± 3.19	ND
Expt 2	1.00 ± 0.50	2.10 ± 0.50	0.0	9.20 ± 1.70	192.2
Expt 3	0.74 ± 0.56	1.56 ± 0.60	ND	8.24 ± 2.56	ND
Expt 4	1.32 ± 0.64	2.50 ± 1.18	ND	23.70 ± 8.60	ND
Fractions					
Expt 1[d]					
Cytoplasmic	0.28 ± 0.06	0.15 ± 0.07	0.0	53.39 ± 6.26	216.0
Membrane		0.80 ± 0.13	0.0	43.76 ± 5.38	190.0
Expt 2[b]					
Cytoplasmic	1.22 ± 0.07	ND	ND	25.20 ± 5.34	ND
Membrane		ND	ND	32.70 ± 7.15	ND
Expt 3[b]					
Cytoplasmic	0.40 ± 0.16	ND	ND	17.21 ± 1.67	ND
Membrane		ND	ND	17.20 ± 2.00	ND

[a] TNF-α values are means ± standard errors of the means (n = 3).
[b] Assayed in serum-free medium.
[c] ND, not done.
[d] Assayed in medium plus 10% FCS.

To determine the optimum concentration of material needed to induce TNF secretion, various doses of Triton X-114 detergent-phase *M. fermentans* particulate proteins were tested. Table 3 shows that concentrations as low as 10 ng/ml are able to induce low levels of TNF. Increasing levels up to 1.0 μg/ml induced increasing levels of TNF. Further increases of the protein concentration to 2.5 μg/ml or higher resulted in lower levels of TNF secretion.

Serum component(s) enhances the level of TNF released by monocytes. To determine whether a serum component was important in the monocyte response to the mycoplasma proteins, TNF-α secretion was measured in the presence and absence of 10% FCS. Table 4 shows that the addition of serum to the monocyte assay increased the level of TNF secretion two- to fourfold in response to *M. fermentans* lysate material. The differences in the levels of TNF produced in the individual experiments reflect the differences in donor responsiveness. Thus, like LPS, a serum component(s) heightens the amount of TNF released by *M. fermentans*.

***M. fermentans* lysate material does not stimulate monocytes through CD14.** To determine whether CD14 was involved in the activation of the monocytes by mycoplasmas, we conducted the following experiment. Monocytes were preincubated with anti-CD14 monoclonal antibody for 1 h before the addition of stimulants (LPS, 100 ng/ml; *M. fermentans* Triton X-114 detergent phase, 1 μg). Monocytes were stimulated for 6 h and the supernatants were harvested and assayed for TNF by using the L929 bioassay. Table 4 shows that preincubation of monocytes with anti-CD14 blocked 60 to 70% of the TNF induced by LPS but had no effect on TNF release stimulated by mycoplasma proteins. These results further demonstrate that the stimulation of TNF release is not due to LPS contamination and that induction proceeds through the activation of a different receptor.

The cytokine-inducing protein has a molecular mass of 48 kDa. To determine the precise molecular weight of the inducing material, 1- to 2-mm strips were assayed by the monocyte Western blot for TNF activity. A broad protein band that partitioned into the detergent phase was left intact, and, therefore, the detergent phase consisted of fewer strips. Table 5 shows that the cytokine-inducing activity was found in the detergent phase of both the soluble and particulate fractions and in the strip containing the major protein band. The molecular mass overlap of the two fractions with the greatest activity is 47 to 48 kDa. There is a small amount of cytokine production (<5 to 10%) induced by the strips flanking this band. IL-1β determinations were done on the detergent-phase samples only since previous results had shown that the aqueous phase contained no activity (Table 1). Figure 3 shows the protein profile of the samples and the Triton X-114 detergent phases. While many bands appear in both fractions, there are several that preferentially partition in one phase as opposed to the other. Likewise, a 48-kDa protein in the membrane fraction preferentially partitions into the detergent phase (Fig. 3, lane 6). A protein of this same molecular weight partitions into the detergent phase of the soluble proteins, although the amount seen in this fraction is much less than that observed in the particulate fraction. These results are consistent with those of Fig. 1, which shows that the majority of TNF-inducing activity is preferentially found in the particulate fraction, and of Table 5, which shows the molecular mass of the material to be 48 kDa.

TABLE 2. Partitioning of cytoplasmic and membrane fraction proteins into Triton X-114 aqueous and detergent phases

Sample	Amt of protein (μg) in:		% of total protein in:	
	Aqueous phase	Detergent phase	Aqueous phase	Detergent phase
Cytoplasmic				
Expt 1	56.40	11.99	82	18
Expt 2	40.10	11.50	78	22
Expt 3	77.91	31.17	71	29
Membrane				
Expt 1	16.69	53.41	24	76
Expt 2	11.80	23.60	32	68
Expt 3	18.60	70.00	21	79

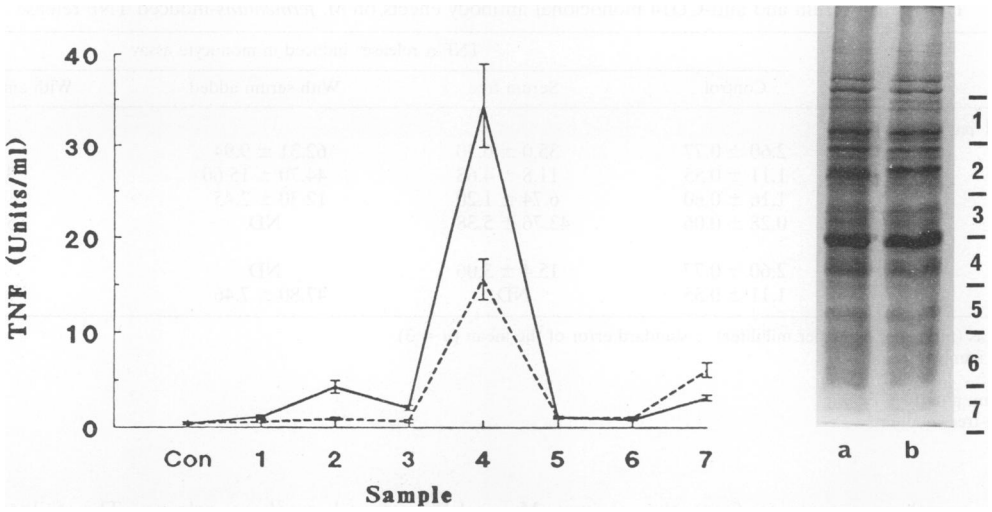

FIG. 2. Lipase treatment effects on TNF-releasing activity. *M. fermentans* membrane fraction was treated with lipoprotein lipase in the presence of 1 mM phenylmethylsulfonyl fluoride and 1 μM leupeptin, separated by SDS–10% PAGE, and blotted onto nitrocellulose. Samples were then assayed for their ability to induce TNF release from normal human monocytes. The solid line represents the untreated sample, and the dashed line represents the lipoprotein lipase-treated sample. Values on the graph are given in mean units of TNF ± standard error of the mean. The molecular weights of the proteins on the nitrocellulose strips are the same as those described in the legend to Fig. 1. The photo insert is a silver-stained gel of the membrane protein sample (lanes: a, untreated; b, lipoprotein lipase treated). The graph is representative of three experiments. Con, control.

DISCUSSION

Mycoplasmas or their products have been shown to produce a number of effects on lymphoid cells and other cultured cell lines. Mycoplasma products have been shown to induce the release of the following cytokines: alpha and gamma interferons, TNF-α, IL-1β, and IL-6 (for a review, see reference 23). This inducing activity can be extremely potent since the levels of TNF-α released by monocytes were found to be greater than that induced by gram-negative endotoxin (11). In this report, we have investigated the ability of *M. fermentans* incognitus to stimulate cytokine secretion by human monocytes and have identified a 48-kDa protein (p48) responsible for this activity. This protein is associated with the particulate or membrane fraction and is very hydrophobic in nature, as is evidenced by its consistent partitioning into the Triton X-114 detergent phase.

The monocyte Western blot is a powerful technique by which to test for cytokine induction and TNF-α-inducing activity. By using this assay, we demonstrated that TNF and IL-1β secretion is induced primarily by a hydrophobic protein in the particulate fraction. While there is activity present in the soluble fraction (<14% particulate) (Fig. 1), we believe that this protein is liberated from the membrane upon sonication of the mycoplasmas. These results are consistent with those of others showing that the membrane fractions of mycoplasma species are responsible for TNF induction (3, 11, 30, 31).

We have extended these previous observations by attributing this activity to a protein with a molecular mass of 48 kDa. Treatment of the mycoplasma fractions with proteinase K removed detectable protein from the samples (as determined by silver staining) and abolished TNF-inducing activity. This demonstrates that the inducing activity is dependent upon a protein moiety. The TNF-inducing activity was found to reside in one fraction with a molecular mass of 41 to 49 kDa. Further analysis of these proteins pinpointed the inducing activity to a protein with a molecular mass of 48 kDa. Triton X-114 partitioning of both the particulate and soluble fractions demonstrates that there is a protein of 48 kDa that preferentially partitions into the detergent phase.

Our results differ from those of others demonstrating cytokine-inducing activity in *M. fermentans.* Muhlradt and Schade (26) and Quentmeier et al. (28) have demonstrated the ability of an *M. fermentans* strain (D15-86) to induce monocytes to secrete TNF, IL-1β, and IL-6. This activity was traced to a high-molecular-mass species of 10^6 Da. In contrast to p48, treatment of their sample with proteinase K did not abolish activity but spread it out over a wider molecular weight range. Likewise, Gallily et al. (11) have demonstrated that strains of *M. fermentans* membrane preparations (KL-4, PG18, IM-1, and incognitus) induce TNF and IL-1β release. However, these were whole-membrane preparations; no molecular weight was attributed to the stimulating moiety, nor was the possibility that other molecules such as lipoglycans were the inducers ruled out. It is important to note that different strains were used in these studies. While each strain had the ability to induce TNF, different strains of *M. fermentans* have been shown to possess variable surface protein profiles (32, 38).

TABLE 3. Concentration effects of *M. fermentans* Triton X-114 detergent-phase proteins on TNF-α induction[a]

Protein concn (μg/ml)	TNF-α induction[b] in expt:		
	1	2	3
0.0	2.37 ± 0.77	2.46 ± 0.78	4.42 ± 1.46
0.01	9.21 ± 2.97	16.82 ± 5.58	10.33 ± 3.16
0.1	51.59 ± 13.39	171.15 ± 53.57	111.64 ± 36.10
1.0	268.33 ± 83.19	616.20 ± 167.24	197.44 ± 59.25
2.5	176.97 ± 44.23	286.16 ± 102.92	153.24 ± 43.85
3.0	96.26 ± 24.98	ND[c]	ND
5.0	ND	230.36 ± 63.91	28.13 ± 7.55

[a] Assays were done in RPMI 1640 plus 10% FCS.
[b] Values are given as (units of TNF-α per milliliter) ± standard error of the mean (n = 3).
[c] ND, not done.

TABLE 4. Serum and anti-CD14 monoclonal antibody effects on *M. fermentans*-induced TNF release

Sample	TNF-α release[a] induced in monocyte assay			
	Control	Serum free	With serum added	With anti-CD14 preincubation
X-114 detergent (1 μg/ml)[b]				
Expt 1	2.60 ± 0.77	35.0 ± 6.30	62.31 ± 9.94	ND[c]
Expt 2	1.11 ± 0.55	11.8 ± 4.63	44.70 ± 15.60	39.28 ± 13.60[d]
Expt 3	1.16 ± 0.60	6.74 ± 1.20	12.30 ± 2.45	6.77 ± 1.38[e]
Expt 4	0.28 ± 0.06	43.76 ± 5.38	ND	54.22 ± 6.25[e]
LPS (100 ng/ml)[b]				
Expt 1	2.60 ± 0.77	15.4 ± 3.06	ND	6.60 ± 1.60[e]
Expt 2	1.11 ± 0.55	ND	47.80 ± 7.46	16.00 ± 2.53[d]

[a] Values are given as (units of TNF-α per milliliter) ± standard error of the mean (n = 3).
[b] Concentration of stimulant.
[c] ND, not done.
[d] Assayed in medium plus 10% FCS.
[e] Assayed in serum-free medium.

Hence, different membrane proteins from the various *M. fermentans* strains may be responsible for the induction of TNF release. Furthermore, it is also probable that additional stimulatory proteins exist but are undetectable by the monocyte Western blot. This technique involves SDS-PAGE, and denaturing of the proteins may result in the loss of activity of the molecule. However, this technique has been used successfully in identifying the cytokine-inducing molecules from mycobacteria (1, 36).

By using the Triton X-114 detergent-phase proteins of the particulate fraction, the optimal concentration for inducing maximum TNF levels appears to be 1 μg/ml. Inductions at higher protein levels resulted in lower levels of TNF secretion. This could be due to the presence of an inhibitor in the mycoplasma preparation that exerts its effect upon reaching a certain level. Likewise, the lower levels of activity may reflect a feedback inhibition produced by the overstimulated monocytes.

Mycoplasma-induced cytokine release was elevated when monocytes were cultured in the presence of serum. Similar results have been reported for LPS and have been attributed to the binding of a serum protein with the endotoxin molecule which then binds to a monocyte/macrophage receptor (10). It is possible that a similar mechanism involving serum protein(s) may direct the mycoplasma lipoprotein to the monocytes. Alternatively, serum may bind or remove a suppressive component from interacting with the monocytes. The monocyte surface molecule CD14 is part of the receptor complex for LPS (12). The addition of monoclonal antibodies to CD14 blocks LPS-induced cytokine release. The failure of monoclonal antibodies to block cytokine induction by mycoplasma material indicates that this induction proceeds through a different receptor. This is supported by the results showing that mycoplasma-induced signal transduction is Ca-dependent while LPS induction is not (33). Mycoplasma membranes contain many lipoproteins (37), and a family of surface lipoproteins has been described in *M. fermentans* (38). Previous reports have shown that bacterial lipoproteins as well as synthetic lipopeptides are good inducers of cytokine secretion from monocytes (13, 24). The lipid moiety of a 17-kDa *Treponema pallidum* membrane protein was found to be essential for TNF-α release by murine macrophages (2). We observed that lipoprotein lipase treatment of p48 removed some but not all cytokine-inducing activity. The protein profiles of treated and untreated samples appear to be similar, with no observable protein degradation. We could not rule out the possibility that there is some residual protease activity that is resistant to phenylmethylsulfonyl fluoride and leupeptin that may account for the loss in activity. However, these results are consistent with previous work that showed that the number of fatty acids is important in the mitogenic effects of lipoproteins. Compounds carrying only one fatty acid are not void of activity but are much less active (5). These results strongly suggest that p48 may be a lipoprotein in that it is dependent upon both protein and lipid moieties for full inducing activity. While the hydrophobic nature and lipoprotein lipase data suggest that the TNF-inducing activity may be the product of a lipid-modified protein, we cannot make a definitive statement as to its nature.

TABLE 5. Molecular mass overlap of cytokine-inducing activity in soluble and particulate fractions

Phase	Cytoplasmic fraction			Membrane fraction		
	Molecular mass (kDa)	TNF-α induced (U/ml)[a]	IL-1β induced (pg/ml)	Molecular mass (kDa)	TNF-α induced (U/ml)[a]	IL-1β induced (pg/ml)
Aqueous	51–49	0.91 ± 0.14	ND[b]	51–49	0.59 ± 0.12	ND
	49–47	0.58 ± 0.13	ND	49–47	1.72 ± 0.18	ND
	47–45	0.44 ± 0.13	ND	47–45	0.68 ± 0.12	ND
	45–43	0.45 ± 0.13	ND	45–43	0.49 ± 0.12	ND
Detergent	51–48	0.86 ± 0.22	32.78	56–53.5	3.67 ± 0.38	158.8
	48–44[c]	20.16 ± 2.29	676.91	53.5–47[c]	41.59 ± 4.28	635.9
	44–41	0.77 ± 0.16	48.1	47–45	1.03 ± 0.12	66.5
Control		0.32 ± 0.13				

[a] Values are means ± standard errors of the mean (n = 3).
[b] ND, not done.
[c] Sample contained a broad protein band that was assayed as a single strip.

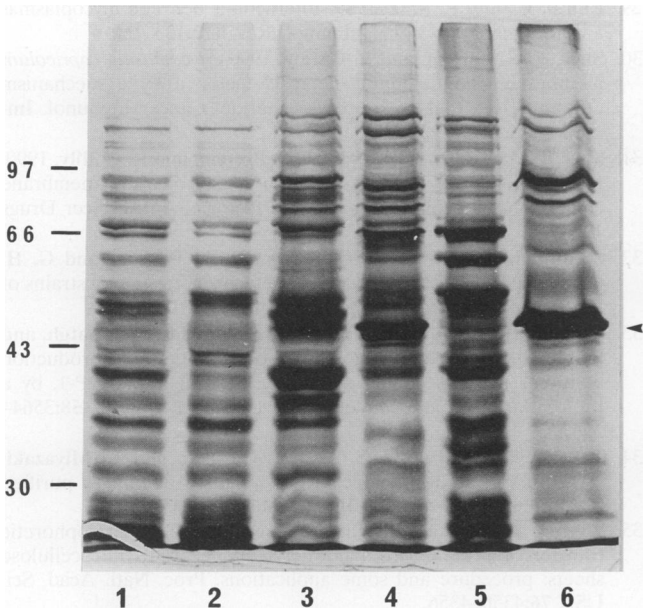

FIG. 3. Triton X-114 partitioning of *M. fermentans* proteins. *M. fermentans* cytoplasmic and membrane fractions were partitioned into aqueous and detergent phases and then separated by SDS–10% PAGE. Proteins were visualized by silver staining. Lanes: 1, cytoplasmic fraction; 2, cytoplasmic fraction, aqueous phase; 3, cytoplasmic fraction, detergent phase; 4, membrane pellet; 5, membrane pellet, aqueous phase; 6, membrane pellet, detergent phase. The arrowhead indicates the band that contains the TNF-inducing activity. It has a molecular mass of 48 kDa. Molecular mass standards in kilodaltons are indicated on the left.

In vivo labelling and cloning of the gene for p48 will ultimately determine the precise nature of the inducing molecule.

Recently, there has been much interest in *M. fermentans* because of its association as a cofactor in AIDS progression. It has been shown to stimulate human immunodeficiency virus replication (8) and is cytotoxic to human polymorphonuclear leukocytes in vitro (18, 20, 22). Additional reports of healthy individuals succumbing to fulminant infections (19) as well as an immunodeficient human immunodeficiency virus-negative individual with a systemic *M. fermentans*-positive infection (21) have raised the question as to its own role as a possible pathogen. Likewise, many of the symptoms described in patients with fulminant *M. fermentans* infections are characteristic of cytokine induction (19). The further identification and characterization of the molecules that induce cytokine secretion such as p48 should help to better understand the mechanism of pathogenicity of this organism.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grant CA53482 and The Donald Guthrie Foundation for Education and Research.

We thank R. E. Hall for critical comments.

REFERENCES

1. **Abou-Zeid, C., E. Filley, J. Steele, and G. A. W. Rook.** 1987. A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes in vitro after converting bands cut from Western blots into antigen-bearing particles. J. Immunol. Methods **98**:5–10.
2. **Akins, D. R., B. K. Purcell, M. M. Mitra, M. V. Norgard, and J. D. Radolf.** 1993. Lipid modification of the 17-kilodalton membrane immunogen of *Treponema pallidum* determines macrophage activation as well as amphiphilicity. Infect. Immun. **61**:1202–1210.
3. **Arai, S., M. Furukawa, T. Munakata, K. Kuwano, H. Inoue, and T. Miyazaki.** 1990. Enhancement of cytotoxicity of active macrophages by mycoplasma: role of mycoplasma-associated induction of tumor necrosis factor-α (TNF-α) in macrophages. Microbiol. Immunol. **34**:231–243.
4. **Beezhold, D. H., J. A. Leftwich, and R. E. Hall.** 1989. P48 induces tumor necrosis factor and Il-1 secretion by human monocytes. J. Immunol. **143**:3217–3221.
5. **Bessler, W. G., E. Simon, and H. Rotering.** 1980. Mitogenicity of a lipid-deficient lipoprotein from a mutant *Escherichia coli* strain. Infect. Immun. **28**:818–823.
6. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256**:1604–1607.
7. **Butler, G. H., H. Kotani, L. Kong, M. Frick, S. Evancho, E. J. Stanbridge, and G. J. McGarrity.** 1991. Identification and characterization of proteinase K-resistant proteins in members of the class *Mollicutes*. Infect. Immun. **59**:1037–1042.
8. **Chowdhury, I. H., T. Munakata, Y. Koyanagi, S. Kobayashi, S. Arai, and N. Yamamoto.** 1990. Mycoplasma can enhance HIV replication in vitro: a possible cofactor responsible for the progression of AIDS. Biochem. Biophys. Res. Commun. **170**:1365–1370.
9. **Cole, B. C., and C. L. Atkin.** 1991. The *Mycoplasma arthritidis* T-cell mitogen MAM: a model superantigen. Immunol. Today **12**:271–276.
10. **Corradin, S. B., J. Mauel, P. Gallay, D. Heumann, R. J. Ulevitch, and P. S. Tobias.** 1992. Enhancement of murine macrophage binding of and response to bacterial lipopolysaccharide (LPS) by LPS-binding protein. J. Leukocyte Biol. **52**:363–368.
11. **Gallily, R., M. Salman, M. Tarshis, and S. Rottem.** 1992. *Mycoplasma fermentans* (incognitus strain) induces TNF-α and IL-1 production by human monocytes and murine macrophages. Immunol. Lett. **34**:27–30.
12. **Heumann, D., P. Gallay, C. Barras, P. Zaech, R. J. Ulevitch, P. S. Tobias, M. P. Glauser, and J. D. Baumgartner.** 1992. Control of lipopolysaccharide (LPS) binding and LPS-induced tumor necrosis factor secretion in human peripheral blood monocytes. J. Immunol. **148**:3505–3512.
13. **Hoffmann, P., K. H. Wiesmuller, J. Metzger, G. Jung, and W. G. Bessler.** 1989. Induction of tumor cytotoxicity in murine bone marrow-derived macrophages by two synthetic lipopeptide analogues. Biol. Chem. Hoppe-Seyler **370**:575–582.
14. **Kirchner, H., G. Brehm, W. Nicklas, R. Beck, and F. Herbst.** 1986. Biochemical characterization of the T-cell mitogen derived from *Mycoplasma arthritidis*. Scand. J. Immunol. **24**:245–249.
15. **Kostyal, D. A., D. H. Beezhold, and R. E. Hall.** 1991. Differentiation inducing cytokine P48 exists in a membrane associated form. J. Immunol. **147**:893–898.
16. **Krause, D. C., and D. Taylor-Robinson.** 1992. Mycoplasma which infect humans, p. 417–444. *In* J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), Mycoplasma: molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lemaitre, M., Y. Henin, F. Destouesse, C. Ferrieux, L. Montagnier, and A. Blanchard.** 1992. Role of mycoplasma infection in the cytopathic effect induced by human immunodeficiency virus type 1 in infected cell lines. Infect. Immun. **60**:742–748.
19. **Lo, S. C., M. S. Dawson, P. B. Newton, M. A. Sonoda, J. W. K. Shih, W. F. Engler, R. Y. H. Wang, and D. J. Wear.** 1989. Association of the virus-like infectious agent originally reported in patients with AIDS with acute fatal disease in previously healthy non-AIDS patients. Am. J. Trop. Med. Hyg. **41**:364–376.
20. **Lo, S. C., S. Tsai, J. R. Benish, J. W. Shih, D. J. Wear, and D. M. Wong.** 1991. Enhancement of HIV-1 cytocidal effects in CD4+ lymphocytes by the AIDS-associated mycoplasma. Science **251**: 1074–1076.
21. **Macon, W. R., S. C. Lo, B. J. Poiesz, D. C. Montefiori, M. S. Dawson, T. E. Mason, P. R. Michael, A. G. Cohen, C. M. Davis,**

J. B. Cousar, and C. W. Stratton. 1993. Acquired immunodeficiency syndrome-like illness associated with systemic Mycoplasma fermentans infection in a human immunodeficiency virus-negative homosexual man. Hum. Pathol. **24:**554–558.

22. **Marshall, A., R. J. Miles, and L. Richards.** 1993. Contrasting effects of *Mycoplasma fermentans* and *M. felis* on the viability and chemiluminescence response of human polymorphonuclear leukocytes. FEMS Microbiol. Lett. **109:**167–172.
23. **McGarrity, G. J., H. Kotani, and G. H. Butler.** 1992. Mycoplasmas and tissue culture cells, p. 445–454. *In* J. Maniloff, R. N. McElhanney, L. R. Finch, and J. B. Baseman (ed.), Mycoplasmas: molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.
24. **Metzger, J., G. Jung, W. G. Bessler, P. Hoffmann, M. Strecker, A. Lieberknecht, and U. Schmidt.** 1991. Lipopeptides containing 2-(palmitoylamino)-6,7bis(palmitoyoxy)heptanoic acid: synthesis, stereospecific stimulation of B-lymphocytes and macrophages, and adjuvanticity in vivo and in vitro. J. Med. Chem. **34:**1969–1974.
25. **Miyazaki, K., H. Takaku, M. Umeda, T. Fujita, W. Huang, T. Kimura, J. Yamashita, and T. Horio.** 1990. Potent growth inhibition of human tumor cells in culture by arginine deiminase purified from a culture medium of a Mycoplasma-infected cell line. Cancer Res. **50:**4522–4527.
26. **Mühlradt, P. F., and U. Schade.** 1991. MDHM, a macrophage-stimulatory product of *Mycoplasma fermentans*, leads to in vitro interleukin-1 (IL-1), IL-6, tumor necrosis factor, and prostaglandin production and is pyrogenic in rabbits. Infect. Immun. **59:**3969–3974.
27. **Proust, J. J., M. A. Buchholz, and A. A. Nordin.** 1985. A "lymphokine-like" soluble product that induces proliferation and maturation of B cells appears in the serum-free supernatant of a T cell hybridoma as a consequence of mycoplasmal contamination. J. Immunol. **134:**390–396.
28. **Quentmeier, H., E. Schmitt, H. Kirchhoff, W. Grote, and P. F. Muhlradt.** 1990. *Mycoplasma fermentans*-derived high-molecular-weight material induces interleukin-6 release in cultures of murine macrophages and human monocytes. Infect. Immun. **58:**1273–1280.
29. **Ruuth, E., and F. Praz.** 1989. Interactions between mycoplasmas and the immune system. Immunol. Rev. **112:**133–160.
30. **Sher, T., S. Rottem, and R. Gallily.** 1990. *Mycoplasma capricolum* membranes induce tumor necrosis factor a by a mechanism different from that of lipopolysaccharide. Cancer Immunol. Immunother. **31:**86–92.
31. **Sher, T., A. Yamin, M. Matzliach, S. Rottem, and R. Gallily.** 1990. Partial biochemical characterization of spiroplasma membrane component inducing tumor necrosis factor a. Anti-Cancer Drugs **1:**83–87.
32. **Stadtlander, C. T. K. H., C. Zuhua, H. L. Watson, and G. H. Cassell.** 1991. Protein and antigen heterogeneity among strains of *Mycoplasma fermentans*. Infect. Immun. **59:**3319–3322.
33. **Sugama, K., K. Kuwano, M. Furukawa, Y. Himeno, T. Satoh, and S. Arai.** 1990. Mycoplasmas induce transcription and production of tumor necrosis factor in a monocytic cell line, THP-1, by a protein kinase C-independent pathway. Infect. Immun. **58:**3564–3567.
34. **Takaku, H., M. Takase, S. Abe, H. Hayashi, and K. Miyazaki.** 1992. In vivo anti-tumor activity of arginine deiminase purified from *Mycoplasma arginini*. Int. J. Cancer **51:**244–249.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4356.
36. **Wallis, R. S., M. Amir-Tahmasseb, and J. J. Ellner.** 1990. Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins: the monocyte Western blot. Proc. Natl. Acad. Sci. USA **87:**3348–3352.
37. **Wieslander, A., M. J. Boyer, and H. Wroblewski.** 1992. Membrane protein structure, p. 93–112. *In* J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), Mycoplasma: molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.
38. **Wise, K. S., M. F. Kim, P. M. Theiss, and S. C. Lo.** 1993. A family of strain-variant surface lipoproteins of *Mycoplasma fermentans*. Infect. Immun. **61:**3327–3333.