Arginine Deiminase of *Mycoplasma hominis*: Cytoplasmic and Membrane-associated Forms

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Membrane and cytoplasmic fractions of *Mycoplasma hominis* inhibited the multiplication of this mycoplasma. Arginine deiminase (EC 3.5.3.6), isolated from both fractions, reproduced the inhibition. The purified cytoplasmic deiminase had a subunit M_r of 49 000, a specific activity of 53 units (mg protein)⁻¹ and an A_{280}/A_{260} ratio of 1.76. The membrane-associated enzyme had an identical M_r but lower values for specific activity [39 units (mg protein)⁻¹] and the A_{280}/A_{260} ratio (1.46). In experiments *in vitro*, recent clinical isolates of *M. hominis* produced less arginine deiminase, but grew faster than the laboratory reference strain PG 21. In addition, other growth inhibitory components associated with membrane preparations were detected in recent clinical isolates but were absent from strain PG 21.

INTRODUCTION

In a previous study, cell surface antigens of *Mycoplasma hominis* were analysed by cross-absorption (Lin, 1980). Membrane preparations obtained from seven reference strains of this mycoplasma were used to absorb antisera prepared against whole cells; these preparations, at certain concentrations, consistently inhibited the growth of the homologous and heterologous strains of *M. hominis*. The present paper reports a further investigation of this phenomenon and shows the presence of cytoplasmic and membrane-associated arginine deiminase (EC 3.5.3.6) which inhibit the multiplication of *M. hominis*.

METHODS

M. hominis strains and antisera. The reference strains for seven serotypes of *M. hominis* were strain PG 21 and six others, isolated and purified in this laboratory (Lin *et al.*, 1975). Strain PG 21 had been used as the laboratory reference strain for *M. hominis* and the number of passages *in vitro* was not clear; three strains of PG 21 from different sources (ATCC 23114, NIH-Chanock, NIH-Baril) were used in the fourth passage from the stock strain received. All other strains were used in the fourth or the fifth passages after the initial isolation and purification and were considered as recent clinical isolates (fewer than 10 passages *in vitro*). Their initial isolates, stored at -70 °C, were also available.

Strains were grown at 37 °C in modified Hayflick's medium and were stored at -70 °C. The titre of organisms was expressed as 50% colour change units (CCU_{50%}) (Lin & Kass, 1975). Antisera against whole cells for each strain of mycoplasma, and control sera from animals immunized with uninoculated Hayflick's medium, were prepared in rabbits as described by Lin *et al.* (1975). Antisera to two other arginine-utilizing species, *M. salivarium* and *M. orale*, were purchased from Microbiological Associates.

Measurements of growth. *M. hominis* strains were grown at 37 °C in modified Edward's broth without deoxyribonucleate (Razin & Rottem, 1976), supplemented with 4% (v/v) horse serum, 12 mM-L-arginine and 0.002% (w/v) phenol red; the pH was adjusted to 6.8. Actively growing cells harvested in the early exponential phase in Hayflick's medium were inoculated into prewarmed Edward's broth at 1/1000 dilutions. Samples were harvested at the indicated times at 37 °C. The pH of the harvested culture was compared with a set of colour references made with a sterile, uninoculated broth; titres of the mycoplasmas were determined in Hayflick's medium. Protein was determined by the Lowry method using crystalline bovine serum albumin as a reference.

Abbreviations: CCU, colour change units; GI, growth inhibition.

Arginine deiminase activity was assayed by the method of Archibald as modified by Ratner (1955); one unit of enzyme activity was defined as the formation of $1 \mu\text{mol}$ citrulline min^{-1} at 37°C . Whole cells were disrupted by freeze-thawing five times before the determination.

Preparations of cytoplasmic and membrane fractions. Overnight cultures in Edward's broth (pH 7.0–7.2) were harvested by centrifuging at $10000 g$ for 30 min. They were then washed twice at 5°C with sterile 0.25 M-NaCl , suspended in 0.25 M-NaCl , concentrated to 1/100 the volume of the original culture, and disrupted by sonication as described by Weickman & Fahrney (1977). Concentrated cell preparations were streaked on blood agar plates which were incubated at 37°C to detect bacterial contamination. Membranes were collected by centrifuging at $45000 g$ for 45 min. They were then washed consecutively with deionized water, 0.05 M-NaCl in $0.01 \text{ M-phosphate-buffered saline (PBS)}$, pH 7.2, and deionized water (Razin & Rottem, 1976). The original supernatants, further centrifuged at $180000 g$ for 1 h, were considered as cytoplasmic fractions. They were concentrated with an Amicon concentrator (B15) to $10 \text{ mg protein ml}^{-1}$, if necessary. Both preparations were stored at -70°C .

Purification of arginine deiminase. The enzymes were isolated from cytoplasmic fractions ($10 \text{ mg protein ml}^{-1}$) by protamine sulphate fractionation (1 mg ml^{-1} ; Weickman & Fahrney, 1977). Supernatant from the fractionation (2–3 ml) was applied directly to an Ultrogel column ($1.6 \times 87 \text{ cm}$, AcA 44, LKB), and was eluted with 0.01 M-PBS , pH 7.2 (flow rate $2\text{--}3 \text{ ml h}^{-1}$). A_{280} and precipitin reactions to antiserum PG21 (immunodiffusion plate, pattern B, Hyland) and determined for each 2 ml fraction. Protein and arginine deiminase activity were determined from A_{280} peak fractions and/or those showing precipitin reactions.

Active fractions were combined, concentrated with Amicon B15 concentrators to 1–2 ml, rechromatographed on the same column and eluted with 0.025 M-Tris/HCl buffer, pH 9.0. If the specific activities of the enzyme fractions were more than 50 units (mg protein^{-1}), they were considered to be pure (Weickmann *et al.*, 1978) and were stored at -70°C .

To extract enzymes from membrane preparations, Tween 20 was added to the preparations ($4\text{--}5 \text{ mg protein ml}^{-1}$) in 0.02 M-Tris/HCl buffer, pH 9.0; the preparations were then shaken at room temperature for 2 h and centrifuged at $180000 g$ for 2 h (Johansson *et al.*, 1975). The supernatant (1 ml) was then chromatographed, firstly on an Ultrogel AcA 34 column ($1 \times 53 \text{ cm}$) with PBS (pH 7.2) to remove the detergent, and then on an Ultrogel AcA 44 column with Tris/HCl buffer, pH 9.0, to purify enzymes.

Homogeneity of the purified enzymes was ascertained by immunoelectrophoresis and gel electrophoresis. Disc polyacrylamide gel electrophoresis was done in the presence of 1% SDS (Fairbanks *et al.*, 1971). The M_r of purified enzymes was estimated with a reference kit (Pharmacia), which contained the following standards: thyroglobulin (M_r 669000), ferritin (440000), catalase (232000), lactate dehydrogenase (140000) and albumin (67000). Immunoelectrophoresis (LKB kit) was done with the purified enzyme against the homologous and heterologous antisera prepared against whole cells.

Growth inhibition. Twofold serial dilutions of material with deiminase activity (0.05 ml) were made in Hayflick's medium in the microtitre system (Falcon) and, unless otherwise stated, $10\text{--}100 \text{ CCU}_{50\%}$ of the mycoplasmas in 0.15 ml of the medium were added to each well. Plates were sealed and incubated at 37°C . The highest dilution which prevented the pH changes of the medium after 5 d was considered as containing 1 growth inhibition (GI) unit (Lin & Kass, 1975).

RESULTS

Growth and production of arginine deiminase

In modified Edward's medium, there were differences in growth and enzyme production between strain PG 21 and the other six strains, as represented by strain 93 (Table 1). Early exponential phase growth was reached at pH 7.0–7.2, and it took more than 24 h for strain PG 21 to reach that phase compared to $\leq 20 \text{ h}$ for strain 93. Strain PG 21 produced 2–5 times more enzyme with a constant rate of production. On the other hand, the rate of enzyme production for strain 93 increased gradually from early to late exponential phase as reflected in increasing specific activities.

Approximately 45% of the cellular proteins and 95% of the deiminase activity were recovered in the cytoplasmic fraction. Although membrane preparations consistently showed low deiminase activity (Table 1), there were again differences between strains PG 21 and 93. Membrane preparations from strain PG 21 had 2–3 times more deiminase activity, but lower GI activity, than those from strain 93. When the GI activities were compared with the deiminase activities, it was found that strain PG 21 had 30–50 GI units ($\text{deiminase unit}^{-1}$) whereas strain 93 had 300–500 GI units ($\text{deiminase unit}^{-1}$). No such differences were observed for cytoplasmic fractions (Table 2). As shown below, purified membrane enzymes from both strains had the

Table 1. *Growth and production of arginine deiminase in M. hominis*

M. hominis strains were grown in modified Edward's broth and incubated at 37 °C. At the indicated times, samples were harvested and activities were determined: titre = 50% colour change unit (CCU_{50%}) ml⁻¹; protein = mg l⁻¹; enzyme = deiminase units (mg cell protein)⁻¹. Strain PG 21 is the original laboratory reference strain and strain 93, representative of the other six reference strains, is a recent clinical isolate.

Time of incubation (h)	pH		Titre		Protein		Enzyme			
							Cell		Membrane	
	PG 21	93	PG 21	93	PG 21	93	PG 21	93	PG 21	93
0	6.8		10 ^{4.8}	10 ^{4.0}						
16	6.8	7.0	10 ^{7.0}	10 ^{7.8}	2.0	10.0	5.27	0.87		0.08
20	6.8	7.2	10 ^{7.3}	10 ^{8.8}	4.9	47.4	5.03	1.11	0.2	0.12
24	7.0	7.4	10 ^{7.8}	10 ^{8.5}	25.3	92.0	5.10	1.87	0.45	0.22
26	7.2	7.6	10 ^{8.3}	10 ^{8.5}	47.5	91.0	5.07	1.87	0.65	0.28
30	7.3	7.8	10 ^{8.0}	10 ^{7.8}	52.2	64.5	5.03	2.76	0.63	0.27
34	7.5	8.1	10 ^{8.0}	10 ^{6.8}	85.3	33.3	5.87	2.54	0.78	0.24
16-24*	7.0-7.2		10 ^{8.0}	10 ^{8.5}	35.9 ± 1.8	41.5 ± 4.6	5.05 ± 0.04	1.55 ± 0.29	0.49 ± 0.1	0.16 ± 0.06

* Combined data for three strains of PG 21 and six reference strains of *M. hominis*; mean ± SD.

Table 2. *Growth inhibiting activities of membrane and cytoplasmic fractions of M. hominis*

Mycoplasmas (10-100 CCU_{50%}) were grown in Hayflick's medium with twofold serial dilutions of test material. The highest dilution which prevented the pH changes of the medium after 5 d incubation at 37 °C was considered as containing 1 GI unit.

Strain	GI units (deiminase unit) ⁻¹	
	Membrane	Cytoplasm
PG 21*	41 ± 6	40 ± 7
93*	382 ± 41	49 ± 8
93 (Original isolate)	336	29
Other six strains	278 ± 60	33 ± 8

* Results of 4 different preparations; mean ± SD.

same values for GI units (deiminase unit)⁻¹, indicating the presence of additional growth-inhibitory components in strain 93.

Initial isolates of two reference strains (93 and 132) stored at -70 °C were recovered and tested. When cells were harvested in exponential phase (pH 7.0-7.2), the patterns of production of arginine deiminase and GI activities in the membrane and cytoplasmic fractions were similar to those of the recent clinical isolates, within the ranges indicated above (Tables 1 and 2).

Cytoplasmic enzyme

Purification of arginine deiminase from the cytoplasmic fraction of strains PG 21 and 93 is summarized in Fig. 1 and Table 3. The specific activities of the purified enzymes were 55 and 51 units (mg protein)⁻¹ respectively, representing 5- to 13-fold increases in the activity. It should be noted that the values for GI units (deiminase unit)⁻¹ of the preparations remained almost constant, between 32 and 42, after each step of the purification procedure.

Purified enzymes showed (1) one major polypeptide band in polyacrylamide gel electrophoresis, with a subunit *M_r* of 49 000 (*R_f* 0.61 ± 0.01) Fig. 2; (2) one precipitin line in immunoelectrophoresis and immunodiffusion against the homologous and heterologous antisera prepared against whole cells; (3) growth inhibition towards the homologous and heterologous

Fig. 1. Isolation of arginine deiminase by Ultrogel AcA 44 chromatography. Cytoplasmic fractions (2–3 ml), obtained after protamine sulphate precipitation, and containing approximately 200 and 80 units of enzyme from strains PG 21 (○) and 93 (●) respectively, were loaded on the column (1.6 × 87 cm) and eluted with 0.01 M-PBS, pH 7.2 (flow rate 2 ml h⁻¹; fraction vol. 2 ml). —, A_{280} ; ····, arginine deiminase activity.

Table 3. *Purification of cytoplasmic deiminase from M. hominis*

Cytoplasmic fractions (10 mg protein ml⁻¹) were extracted with protamine sulphate and chromatographed on Ultrogel AcA 44 columns with 0.01 M-PBS, pH 7.2. Active fractions were rechromatographed with 0.015 M-Tris/HCl buffer, pH 9.0.

Strain	Step	Protein (mg)	Specific activity [units (mg protein) ⁻¹]	Total activity (units)	Yield (%)
PG 21	Extract	18.0	11.2	201.6	100
	Chromatography	5.6	33.5	187.6	93.1
	Rechromatography	1.4	55.0	77.0	38.2
93	Extract	21	3.9	81.9	100
	Chromatography	4.8	16.5	79.2	96.7
	Rechromatography	0.7	50.8	35.6	43.5

strains; (4) A_{280}/A_{260} ratios of 1.74 and 1.78. Among the seven reference antisera, immunoprecipitation was strongest for strain PG 21. The other six reference antisera, and antisera to *M. salivarium* and *M. orale*, showed weak reactions. Control antisera showed no activity.

Membrane-associated deiminase

When washed membranes were extracted with Tween 20, all the deiminase activity was recovered in the Tween 20 fractions. Approximately 45% and 25% of the membrane proteins were solubilized from strains PG 21 and 93 respectively. Deiminase purified from membranes of strains PG 21 and 93 had an R_F of 0.6 (Fig. 2), 37.7 and 40.1 deiminase units (mg protein)⁻¹, 35.1 and 30.8 GI units (deiminase unit)⁻¹, and A_{280}/A_{260} ratios of 1.40 and 1.52, respectively.

Because of the low deiminase content of membrane preparations, active fractions were much

Fig. 2. SDS-polyacrylamide disc gel electrophoresis of preparations from *M. hominis* strain PG 21. A, Membrane preparations (110 µg proteins); B, membrane arginine deiminase (10 µg); C, cytoplasmic preparations (150 µg); D, cytoplasmic arginine deiminase (30 µg).

Table 4. Growth inhibiting capacity of arginine deiminase in relation to *M. hominis* cell number

Twofold serial dilutions of purified arginine deiminase from strain PG 21 were incubated with 10-fold serial dilutions of cells from strain PG 21 in microtitre plates. The highest dilutions of enzyme which inhibited the growth of the mycoplasma were recorded. Preparation A contained 3.8 deiminase units, and B 7.1 deiminase units.

Cell titre (CCU _{50%} ml ⁻¹)	GI units (deiminase unit) ⁻¹		GI units (deiminase unit) ⁻¹	
	A	B	A	B
10 ^{8.3} –10 ^{7.3}	2.1	2.3	0.48	0.44
10 ^{6.3} –10 ^{5.3}	4.2	9.1	0.24	0.11
10 ^{4.3} –10 ^{3.3}	8.4	16.6	0.12	0.06
10 ^{2.3} –10 ^{1.3}	16.8	35.2	0.06	0.03

more difficult to identify by using A_{280} ; they were identified by the precipitin reaction and/or by GI. Protein and deiminase activity were often determined after active fractions were concentrated 5–10 times. From 60–75 mg of membrane proteins, approximately 0.2 and 0.1 mg enzyme were purified from strains PG 21 and 93, respectively (38% and 28% recovery).

Growth inhibition

GI values of purified enzyme preparations were inversely related to the concentrations of mycoplasmal cells being tested; 0.5 units of deiminase inhibited $\leq 10^{8.3}$ CCU_{50%} of the mycoplasma (Table 4). Both crude and purified enzyme preparations from strains PG 21 and 93 inhibited cultures of strains PG 21 and 93 at 0.5 units deiminase ml⁻¹; the mycoplasma

Table 5. *Growth inhibition of M. hominis by crude cytoplasmic and membrane preparations*

Strain PG 21 in Hayflick's medium was incubated with homologous preparations at 37 °C. Arginine deiminase activity in the reaction mixtures was 0.5 units ml⁻¹.

Time at 37 °C (h)	Control	Mycoplasma titres (CCU _{50%} ml ⁻¹)	
		+ Cytoplasmic fraction*	+ Membrane fraction
0	10 ^{6.1}		
4	10 ^{6.1}		
24	10 ^{8.8}	10 ^{5.8}	10 ^{6.1}
30	10 ^{8.5}	10 ^{4.8}	10 ^{5.6}
48	10 ^{7.3}	10 ^{3.6}	10 ^{3.1}
72	10 ^{3.1} (>8.0)†	10 ^{1.8} (7.2)†	10 ^{2.6} (7.0)†

* Purified cytoplasmic deiminase from strain PG 21 had the same effect.

† Final culture pH shown in parentheses.

remained viable in the growth medium for at least 2 d but failed to multiply in the presence of enzyme preparations (Table 5). The pH of the inhibited culture remained low (<8.0), suggesting that arginine was not depleted but that the metabolism of arginine was inhibited in the medium.

DISCUSSION

Prokaryotes metabolize arginine by the arginine dihydrolase pathway (Razin, 1978);

Arginine-utilizing strains of mycoplasmas are particularly toxic to mammalian cells in culture. Soluble extracts of these mycoplasmas inhibit lymphocyte mitosis, antibody production and the multiplication of mammalian cells in culture; partially purified arginine deiminases from mycoplasmas, bacteria and yeasts reproduce these effects (Simberkoff *et al.*, 1969; Gill & Pan, 1970). Large amounts of the arginine dihydrolase pathway enzymes were present in a laboratory reference strain of murine mycoplasma, *M. arthritidis*, grown in an artificial medium, and arginine deiminase constitutes about 10% of the cytoplasmic proteins (Schimke *et al.*, 1966; Weickmann *et al.*, 1978). The results reported here for the laboratory reference strain PG 21 of the human genital mycoplasma, *M. hominis*, are similar. In addition, membrane-associated arginine deiminase has been detected and isolated from this strain. However, recent or original clinical isolates of *M. hominis* have been shown to contain less of the enzymes than the laboratory reference strain.

The presence of cytoplasmic and membrane-associated arginine deiminases in *M. hominis* suggests multiple metabolic roles for arginine in the non-glycolytic, arginine-utilizing mycoplasmas. It has been suggested that the arginine dihydrolase pathway is a major source of energy, but recent results suggest that arginine is used in *M. hominis* as an alternative energy source (Fenske & Kenny, 1976). On the other hand, the pathway might be required for active

transport of amino acids (Razin *et al.*, 1968), a possibility supported by the presence of membrane-associated arginine deiminase. Presumably, the other two enzymes in the dihydrolase pathway would be expected to be present in membranes of *M. hominis*.

Two distinct forms of arginine deiminase have been identified in the cytoplasm of *M. arthritidis*; deiminase I is found only in exponential phase cultures and deiminase II accounts for 30–60% of the enzyme from late exponential phase cultures (Weickmann *et al.*, 1978). The two forms have identical subunit sizes (M_r 49000) but deiminase II has lower values for specific activity [55 and 44 units (mg protein)⁻¹ and for the A_{280}/A_{260} ratio (1.5 compared to 1.8). The results presented here show that the cytoplasmic arginine deiminase from *M. hominis* resembles deiminase I in physical properties and that the membrane-associated enzyme resembles deiminase II. The membrane-associated deiminase appears to be a peripheral protein since it can be extracted by Tween 20. Unfortunately, Weickmann *et al.* (1978) did not determine deiminase activity in the membrane, so more direct comparison with the present work to determine the intracellular localization of deiminase II is not possible.

In the experimental conditions reported here, the minimum amount of mycoplasma arginine deiminase required to inhibit the multiplication of *M. hominis* was 0.03–0.06 units ml⁻¹ (Table 4). These figures are comparable to the 0.02 units of *Streptococcus* enzymes required to inhibit lymphocyte mitosis, and the 0.05 units of *M. orale* enzymes and the 0.03 units of yeast enzymes required to inhibit cell division of a leukaemic cell line (Simberkoff *et al.*, 1969; Gill & Pan, 1970). Thus, it appears that cells requiring arginine for growth are susceptible to the inhibitory effects of arginine deiminases from various sources. The possibility exists that release of the enzyme *in vivo* may play a role in the pathogenesis of diseases caused by *M. hominis*. On the other hand, some potent growth-inhibiting components are associated with membranes of recent clinical isolates but absent from the laboratory reference strain PG 21. Further studies are needed to identify these components and to determine whether they play a role in pathogenesis.

Recent clinical isolates of *M. hominis* produced less arginine deiminase, in both membranes and cytoplasm, but grew faster than the laboratory reference strain PG 21 under the experimental conditions used. The finding of growth inhibitory components other than the deiminase in membranes of recent clinical isolates but not in strain PG 21, and previous results showing that strain PG 21, among the seven reference strains of *M. hominis* examined, had the fewest antigens expressed at the cell surface (Lin, 1980), indicate that recent clinical isolates of *M. hominis* are biochemically different from laboratory-adapted strains and that losses of 'virulence' factors may occur during laboratory passages.

Understanding the mechanism of growth inhibition may help to define the metabolic and pathogenic role of arginine deiminase. Preliminary results suggest that attachment of arginine deiminases to mycoplasma cells may inhibit the metabolism of arginine and, concurrently, multiplication of the mycoplasma, since live mycoplasma cells or membrane preparations in non-growth media absorbed deiminase and the corresponding growth inhibiting activities from the cytoplasmic fractions. It is also possible that reactive sites for deiminase and growth inhibition may be different. An antiserum to the purified cytoplasmic enzyme of strain PG 21 has been prepared; at a 1:4 dilution this antiserum neutralized >90% of the cytoplasmic arginine deiminase (2 units) and growth inhibiting (128 GI units) activities of strain PG 21. It also neutralized 70% of the deiminase activity (1 unit) in membranes but failed to affect growth inhibiting activity (64 units). Further studies are needed to understand the mechanism of growth inhibition. Preparations of monoclonal antibodies to the enzyme may help to define the mechanisms involved in growth inhibition.

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