Aminopeptidase Activity of *Acholeplasma laidlawii*, *Mycoplasma bovirhinis*, *Mycoplasma dispar* and *Mycoplasma bovis*

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Aminopeptidase activity was demonstrated in extracts of *Acholeplasma laidlawii*, *Mycoplasma bovirhinis*, *M. bovis* and *M. dispar*. The enzyme specificity, which differed amongst the mycoplasma species examined, was characterized using 19 aminoacyl- β -naphthylamides as substrates. The differences were detected after 4 h incubation with the substrates and potentially could be employed to aid in the characterization of certain mycoplasma species.

INTRODUCTION

Mycoplasma species are identified primarily on the basis of antigenicity and, to a lesser extent, cultural and biochemical characteristics (Sub-Committee on the Taxonomy of Mycoplasmatales, 1972). The biochemical tests are based on existing bacteriological methods (Aluotto *et al.*, 1970). Proteolytic activity has been recognized in several mycoplasma species (Aluotto *et al.*, 1970; Czekalowski *et al.*, 1973) and specific aminopeptidases have been demonstrated in *Acholeplasma laidlawii* (Choules & Grey, 1971; Pecht *et al.*, 1972), *Ureaplasma urealyticum*, *Mycoplasma pneumoniae*, *M. hominis* and *M. fermentans* (Vinther & Black, 1974). None of these activities has been extensively investigated or taxonomically exploited.

Aminopeptidases have been demonstrated in a variety of bacteria and fungi (Muftic, 1967; Westley *et al.*, 1967; Huber & Mulanx, 1969; Burton *et al.*, 1970; Krawczyk & Huber, 1972; Lee *et al.*, 1975). The substrate specificities of the aminopeptidases of micro-organisms can be used to distinguish closely related species and even strains of the same species (Watson, 1976).

We have now compared the substrate specificities of the aminopeptidases from strains of *A. laidlawii*, *M. bovirhinis*, *M. bovis* and *M. dispar* using 19 commercially available aminoacyl- β -naphthylamide substrates.

METHODS

Organisms. Sixteen strains of mycoplasma were used (Table 1). All laboratory isolates were identified using specific fluorescein-conjugated rabbit antisera raised against the type cultures *A. laidlawii* (NCTC 10116), *M. bovirhinis* (NCTC 10118), *M. dispar* (NCTC 10125) and *M. bovis* (NCTC 10131).

Culture medium. Organisms were grown in Frey's FM-4 medium (Frey *et al.*, 1968) containing 15 % (v/v) serum, pH 7.9. Pig serum was separated from pooled blood collected at a local abattoir and mycoplasma-free lamb serum, horse serum and newborn calf serum were obtained from Gibco Bio-cult, Paisley, Scotland. Sera used in growth media were inactivated at 56 °C for 30 min and stored at –20 °C. Complete medium was stored at 4 °C and used within 3 weeks of preparation.

Preparation of cell extracts. Mycoplasma strains were grown in 200 ml medium incubated at 37 °C. Just before harvesting the organisms the pH of the culture was measured. The organisms were harvested by centrifugation at 40000 g for 30 min, resuspended and washed twice in 0.9 % (w/v) NaCl, and finally resuspended in 2 to 3 ml double-distilled water. Triton X-100 (Sigma) was added to a final concentration of approximately 1 % (v/v) and the suspension was left at 4 °C for 1 h. Coarse particulate material was removed

Table 1. *Organisms used*

Species	Strain	Source*	No. of passages	Growth period (d)	pH value at harvest
<i>Acholeplasma laidlawii</i>	A5	Bovine vaginal mucus; VRLB	14	4	7.3
	NCTC 10116	Sewage	Unknown	2	6.5
	2098	Calf lung; VRLB	16	1	6.4
<i>Mycoplasma bovirhinis</i>	MC22	Calf nasal swab; VRLB	22	2	7.6
	MC38	Calf nasal swab; VRLB	13	2	7.35
	MC48	Calf nasal swab; VRLB	13	1	7.0
	MC17	Calf nasal swab; VRLB	13	1	6.65
	NCTC 10118	Bovine respiratory tract	Unknown	1	6.0
<i>Mycoplasma dispar</i>	NCTC 10125	Calf lung	Unknown	4	7.6
	193	Calf nasal swab; VRLB	33	3	7.4
	3737	Calf lung; VRLB	3	3	7.3
	MC158	Calf lung; VRLB	7	2	6.75
	MC61	Calf lung; VRLB	5	2	6.4
<i>Mycoplasma bovis</i>	170	Calf lung; IRAD	3	3	7.6
	NCTC 10131	Bovine udder	Unknown	3	7.5
	130	Calf lung; IRAD	4	1	7.3

* IRAD, Institute for Research on Animal Diseases, Compton, Newbury; VRLB, Veterinary Research Laboratories, Belfast.

by centrifugation and the resulting supernatant was assayed for enzyme activity. The cell extracts were stored at -20°C if not used immediately. Total protein in the extracts was determined by the method of Hartree (1972).

Chemicals. The β -naphthylamide derivatives of alanine, isoleucine, serine, cystine and leucine (the latter as the hydrochloride) were supplied by Sigma, those of valine, phenylalanine, tyrosine, tryptophan, arginine, lysine (as carbonate), glycine and proline (as hydrobromides) and pyrrolidonyl- β -naphthylamide were obtained from I.C.N. Pharmaceuticals, Cleveland, Ohio, U.S.A.; the derivatives of histidine, 4-methoxy-leucine and 4-hydroxyproline and 1-aspartyl- β -naphthylamide and 5-glutamyl- β -naphthylamide were from Koch-Light. The β -naphthylamides were all derivatives of L-amino acids.

Enzyme assay. Aminopeptidase activity was measured by following the hydrolysis of aminoacyl- β -naphthylamides. Substrates (10^{-4} M) were dissolved in 0.1 M-Tris/HCl buffer, pH 8.0, and then dispensed in 2 ml amounts into glass Bijou bottles, capped and sterilized by autoclaving for 10 min at 121°C . L-Glutamyl- β -naphthylamide, which hydrolyses on autoclaving, was filter-sterilized prior to dispensing. The sterilized substrate solutions were stored at 4°C until required. Extract (0.2 ml) was added to each of the buffered substrate solutions (2 ml) previously warmed to 37°C and incubation was continued. Release of fluorescent β -naphthylamine was measured using a Perkin-Elmer fluorescence spectrophotometer, model 204A (excitation λ 337 nm; emission λ 408 nm). The mean fluorescence of duplicate reaction mixtures was determined after 4 h incubation at 37°C . Substrate instability was corrected for by subtracting the mean fluorescence for the appropriate buffered substrate solutions lacking extract. The arbitrary fluorescence units were converted to μmol β -naphthylamine released, using a linear calibration curve prepared with 5 to 100 μmol β -naphthylamine. Aminopeptidase activity of cell extracts was recorded as μmol β -naphthylamine formed (mg protein^{-1}).

RESULTS AND DISCUSSION

Aminopeptidase activity was demonstrated in all of the mycoplasma extracts examined. Preliminary experiments, using extracts prepared from strains of each species, indicated that the rates of substrate hydrolysis were not constant over a 21 h incubation period. Results are therefore reported for enzyme activities after 4 h incubation with substrates, as the reaction rates for most substrates were constant for this period. It is not known whether extracts contained single non-specific enzymes or several enzymes specific for individual substrates. The aminopeptidase activity of the extracts prepared from different strains of the same species differed quantitatively but not qualitatively when tested with a range of substrates. This is shown for the three strains of *A. laidlawii* (Table 2). A few exceptions were

Table 2. *Aminopeptidase activity of A. laidlawii extracts*

Extracts were prepared from cultures grown in FM-4 medium containing 15 % (v/v) pig serum. Enzyme activities were measured using 4 h incubation (see Methods). The results are the average of duplicate assays on two samples taken from each culture. The period of incubation and the pH of the culture at the time of harvest are shown in parentheses.

Substrate*	Aminopeptidase activity [$\mu\text{mol } \beta\text{-naphthylamine released in 4 h (mg protein)}^{-1}$]		
	Strain A5 (4 d, pH 7.3)	Strain 10116 (2 d, pH 6.5)	Strain 2098 (1 d, pH 6.4)
Ala-ONA	0	35.7	8.65
Val-ONA	5.8	16.7	8.65
Leu-ONA	17.4	102.4	38.5
Ile-ONA	0	2.4	2.4
Phe-ONA	16.3	290.5	73.1
(4-OMe)Leu-ONA	20.9	152.3	42.3
Asp ¹ ONA	0	10.7	9.6
Glu ⁵ ONA	2.3	9.5	2.9
Lys-ONA	2.9	16.7	3.9
Arg-ONA	1.7	13.1	71.2
Ser-ONA	3.5	5.9	0
Tyr-ONA	36.6	178.6	113.5
Trp-ONA	66.3	201.2	111.5
Gly-ONA	0	0	9.6
Cys-ONA	0	0	4.8
Cys-ONA	0	0	4.8
His-ONA	4.7	8.3	4.8
Pro-ONA	3.5	19.1	2.9
(4-OH)Pro-ONA	0	5.6	1.0
Pyr-ONA†	2.3	7.1	0

* -ONA, β -Naphthylamide derivative.

† Pyr-ONA, Pyrrolidonyl- β -naphthylamide.

observed, for example hydrolysis of glycyl- and cystine- β -naphthylamides occurred with extracts of only one of the three cultures. Such exceptions occurred only when relatively low levels of hydrolysis were observed. Extracts prepared from strains of different species varied both quantitatively and qualitatively in the range of substrates hydrolysed (Table 3).

A significant negative correlation ($P < 0.05$) was observed between the pH of the culture at the time of harvesting and the aminopeptidase activity for the five strains of *M. bovirhinis* tested. This relationship was not observed with any of the other species examined. Most of the strains were harvested in the late-exponential or stationary phase of growth and no relationship could be shown between the stage of growth and aminopeptidase activity, or between aminopeptidase activity and the age of the cultures at the time of harvesting.

Aminopeptidase activity of extracts prepared from four cultures of *M. bovirhinis* MC38 grown in FM-4 medium containing calf, sheep, horse, pig serum, respectively, were compared. Activity against individual substrates was altered by using different sera in the growth medium, but the use of any one serum did not lead to increased or decreased activity against all substrates. Changing the serum supplement, which is a critical but variable component of all media used for mycoplasma culture, only altered the extent to which a particular range of substrate solutions was hydrolysed. A similar effect was observed using extracts from the other mycoplasma species.

No hydrolysis was demonstrated using uninoculated FM-4 medium without serum; however, medium containing serum of each type hydrolysed the majority of substrate solutions, hydrolysis varying within the range <1.0 to $17.1 \mu\text{mol } \beta\text{-naphthylamine (mg protein)}^{-1}$. Contamination of washed mycoplasma cell preparations by serum proteins giving rise to non-specific serological reactions has been described by Bradbury & Jordan (1972) and Yaguzhinskaya (1976). It is therefore possible that the proteins responsible for serum

Table 3. *Comparison of the aminopeptidase activities of extracts from four mycoplasma species*

Extracts were prepared from cultures grown in FM-4 medium containing 15 % (v/v) pig serum. Enzyme activities were measured using 4 h incubation (see Methods). The values shown for each species are the means of the activities of extracts from strains within that species, with the standard errors given in parentheses.

Substrate*	Aminopeptidase activity [$\mu\text{mol } \beta\text{-naphthylamine released in 4 h (mg protein)}^{-1}$]			
	<i>A. laidlawii</i>	<i>M. bovirhinis</i>	<i>M. dispar</i>	<i>M. bovis</i>
Ala-ONA	14.7 (10.8)	11.9 (4.1)	8.1 (3.2)	2.9 (1.8)
Val-ONA	10.3 (3.3)	1.6 (0.7)	0.2 (0.1)	0.6 (0.6)
Leu-ONA	52.8 (25.5)	39.8 (18.7)	1.1 (0.4)	2.8 (2.8)
Ile-ONA	1.6 (0.8)	1.2 (0.6)	1.7 (1.5)	0.1 (0.1)
Phe-ONA	126.6 (83.6)	4.2 (2.4)	0.7 (0.7)	0 (0)
(4-OMe)Leu-ONA	71.8 (40.7)	8.5 (0.6)	2.1 (1.7)	0.4 (0.4)
Asp ¹ ONA	10.2 (0.5)	3.1 (1.5)	2.8 (2.8)	2.9 (2.9)
Glu ² ONA	4.9 (2.3)	0.5 (0.2)	0.7 (0.7)	1.0 (0.8)
Lys-ONA	7.8 (4.4)	11.7 (6.2)	1.0 (0.5)	1.5 (0.8)
Arg-ONA	43.0 (27.8)	37.3 (18.3)	3.4 (2.2)	2.5 (1.6)
Ser-ONA	3.1 (1.7)	2.1 (0.7)	2.4 (0.4)	1.4 (0.7)
Tyr-ONA	109.5 (41.0)	2.4 (1.9)	0.8 (0.8)	0 (0)
Trp-ONA	126.3 (39.7)	35.2 (12.9)	1.3 (0.8)	1.8 (1.1)
Gly-ONA	3.2 (3.2)	0.7 (0.4)	0 (0)	0 (0)
Cys-ONA				
	1.6 (1.6)	2.1 (1.2)	1.6 (1.4)	0.3 (0.3)
Cys-ONA				
His-ONA	5.9 (1.2)	45.4 (20.7)	0.2 (0.2)	1.2 (0.9)
Pro-ONA	8.5 (5.3)	0.1 (0.1)	0 (0)	0 (0)
(4-OH)Pro-ONA	2.0 (2.0)	2.6 (1.8)	1.7 (1.0)	0.5 (0.5)
Pyr-ONA†	3.2 (2.1)	0.7 (0.3)	0.9 (0.7)	0.5 (0.5)

* -ONA, β -Naphthylamide derivative.

† Pyr-ONA, Pyrrolidonyl- β -naphthylamide.

aminopeptidase activity may have been adsorbed on to the mycoplasma cell membrane. But whatever the origin of the mycoplasma aminopeptidase activity detected, the range of substrates hydrolysed was constant for strains of one species.

The range of substrates hydrolysed by strains of a species remained the same regardless of the age or final pH of the culture, or the type of serum in the growth medium. For a given species, substrate specificity was represented as the mean of the hydrolytic activities of extracts prepared from all strains of that species. The results (Table 3) indicated major differences in the extent to which particular substrate solutions were hydrolysed by extracts from each species. The extent of hydrolysis always decreased in the order *A. laidlawii* > *M. bovirhinis* > *M. bovis* and *M. dispar*. All of these species have been isolated from pathological material and all have been implicated to varying degrees in calf pneumonia (Thomas & Smith, 1972; Thomas *et al.*, 1975; Brownlie *et al.*, 1976; Gourlay *et al.*, 1976; Howard *et al.*, 1976). This relative decline in aminopeptidase activity is in the direction of increasing pathogenicity generally accredited to each of these species.

The hydrolysis of aminoacyl- β -naphthylamides could have potential value in the characterization and differentiation of mycoplasma species. Certain substrates, for example alanyl- β -naphthylamide, were hydrolysed by all of the species to an extent that is of little or no value for the purposes of differentiation. Phenylalanyl-, methoxyleucyl- and tyrosyl- β -naphthylamides were hydrolysed almost exclusively by *A. laidlawii* and hence are potentially of value in the differentiation of this species from the three *Mycoplasma* species tested. Similarly, the derivatives of leucine, arginine, tryptophan and histidine could be used to differentiate *M. bovirhinis* from *M. dispar* and *M. bovis*. Because of the relatively low levels of hydrolysis exhibited by extracts of *M. dispar* and *M. bovis*, the number of strains of these species tested was inadequate to draw any firm conclusions regarding differentiation between

these two species. The results indicate that the measurement of aminopeptidase activity using a small number of β -naphthylamide derivatives of amino acids promises to be a useful and rapid technique to aid the characterization of certain mycoplasma species.

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