Rapid Biochemical Tests for Characterization of the *Mycoplasmatales*

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Methods are described for the rapid detection of β -D-glucosidase and phosphatase in mycoplasma cultures using fluorogenic 4-methylumbelliferone substrates. These methods were applied to a selection of mycoplasma cultures and were compared with the conventionally used tests for these enzymes. Results were similar by both methods, but the fluorogenic tests could be read after 1 h, whereas the conventional tests took several days.

In 1967 Fabricant and Freundt drew attention to the lack of simple laboratory tests for mycoplasma characterization (4), and since that time a number of serological and biochemical procedures have been developed for the purpose.

The biochemical tests appear to have been adapted from existing bacteriological methods (1), but suffer from the disadvantage that results are obtained relatively slowly. Many of them are designed to detect enzyme activity, for which a suitable substrate is added to the medium, and, after growth, the culture is examined for evidence of substrate breakdown as in the esculin test for β -D-glucosidase (9) and in the phosphatase test (1). In both procedures it may be necessary to incubate cultures for as long as 14 days.

The present study was initiated in an attempt to devise simple and rapid biochemical procedures for mycoplasma characterization, and this communication describes such tests for detecting β -D-glucosidase and phosphatase enzymes using fluorogenic substrates prepared from 4-methylumbelliferone (4MU). The tests are based on the generation of fluorescence when free 4MU is released by enzyme hydrolysis of the substrates, and reactions can be detected after 1 h.

To assess the accuracy of these tests, they were compared with the conventionally used methods for β -D-glucosidase and for phosphatase using a selection of *Acholeplasma* and *Mycoplasma* species.

MATERIALS AND METHODS

Organisms. The strain designation of the organisms and their source are shown in Tables 1 and 2.

Media. (i) **For routine subcultures.** Mycoplasma broth and agar for routine subculture contained

1.47% (wt/vol), PPLO broth powder without crystal violet (Difco) supplemented with 15% (vol/vol) sterile heated swine serum, 10% (vol/vol) of a 25% (wt/vol) yeast extract, 0.01% (wt/vol) nicotinamide adenine dinucleotide, 0.1% (wt/vol) glucose, 1,000 IU of penicillin per ml, and 0.05% (wt/vol) thallium acetate. The agar contained 1% (wt/vol) agar no. 2. (London Analytical and Bacteriological Media Ltd.) and the broth contained 0.002% (wt/vol) phenol red.

(ii) **For conventional biochemical tests.** For the esculin test the agar medium was that described by Williams and Wittler (9) containing 0.1% (wt/vol) esculin and 0.05% (wt/vol) ferric citrate. For phosphatase tests agar was supplemented with sodium phenolphthalein-diphosphate as described (9) except that 15% (vol/vol) swine serum was used in place of 20% horse serum, because this gave better growth of the mycoplasmas which were already adapted to swine serum medium.

Fluorogenic substrates. (i) **For detection of β -D-glucosidase.** Five millimolar 4MU β -D-glucoside (Koch-Light Laboratories Ltd., Buckinghamshire, England) was dissolved in 0.1 M phosphate buffer, pH 7.5.

(ii) **For detection of phosphatase.** (a) Five millimolar 4MU phosphate (Koch-Light Laboratories Ltd.) was dissolved in 0.1 M citrate buffer, pH 4.8. (b) As in (a) but with 5 mM $MgCl_2$.

The pH 4.8 buffer was selected after pilot experiments to compare it with tris(hydroxymethyl)amino-methane buffer (pH 7.5) and glycine buffer (pH 10.5) (6).

Substrate solutions were freshly prepared on the day of use.

Fluorogenic test (Fig. 1). Test cultures were grown on routine agar medium for 48 h to give moderate to heavy colony density. Colony samples were then transferred to a clean glass plate by means of a rubber stopper (2). The glass plate had been subdivided into labeled areas of approximately 2.5 by 2.5 cm with a grease pencil. For each culture under test, two test areas of colony transfers were made to give approximately the amount of material shown in Fig. 2. For the negative control a transfer

TABLE 1. Tests for β -D-glucosidase activity

Species	Strain	Source ^a	Conventional esculin test ^b	Fluorogenic test ^b
<i>Acholeplasma laidlawii</i>	PG8	NIH	+	+
	PG9	NIH	+	+
	S-Algen	CCAM	w+	w+
	T91/L31	CCAM	+	+
	S-Mist	CCAM	w+	+
	BJ29	CCAM	w+	w+
	C881	CCAM	w+	w+
	C884	CCAM	—	—
	M996/75	RHL	—	—
	M1326/75	RHL	+	+
	M2332/75	RHL	+	+
	S743	NCTC	+	+
<i>A. axanthum</i>	B107PA	RHL	+	+
	M1533/71(D3)	RHL	+	+
	H86N	RHL	+	+
	19L	NCTC	+	+
<i>A. oculi</i>	Squire	NCTC	—	—
<i>A. modicum</i>	BTS-39	NCTC	—	—
<i>Mycoplasma gallisepticum</i>	PG31	NIH	—	—
<i>M. gallinarum</i>	PG16	NIH	—	—
<i>M. iners</i>	PG30	NIH	—	—

^a NIH, National Institutes of Health, Md., U.S.A.; CCAM, FAO/WHO Collaborating Centre for Animal Mycoplasmas, Aarhus, Denmark; RHL, R. H. Leach, Mycoplasma Reference Laboratory, Colindale, England; NCTC, National Collection of Type Cultures, Colindale, England.

^b +, Clear positive reaction; w+, weak positive reaction; —, negative reaction.

TABLE 2. Tests for phosphatase activity in mycoplasma species

Species	Strain	Source ^a	Conventional test ^b	Fluorogenic test ^{b, c}
<i>Mycoplasma agalactiae</i> subsp. <i>agalactiae</i>	PG2	NCTC	+	+
<i>M. alkalescens</i>	D12	NCTC	+	+
<i>M. arthritidis</i>	PG6	WL	+	+
<i>M. arthritidis</i>	PG27	NIH	+	+
<i>M. bovigenitalium</i>	PG11	NIH	+	+
<i>M. bovirhinis</i>	PG43	NIH	—	—
<i>M. bovis</i>	Donetta	NCTC	+	+
<i>M. buccale</i>	CH20247	NIH	+	+
<i>M. hyorhinis</i>	BTS-7	NIH	+	+
<i>M. maculosum</i>	PG15	NIH	+	+
<i>M. salivarium</i>	PG20	NIH	—	—
<i>M. spumans</i>	PG13	NIH	+	+

^a NCTC, National Collection of Type Cultures; WL, Wellcome Laboratories (Dr. R. H. Leach); NIH, National Institutes of Health, Md., U.S.A.

^b +, Clear positive reaction; —, negative reaction.

^c Reactions enhanced in presence of Mg^{2+} ions.

was made from a sterile agar plate and was situated between the duplicate test cultures. A known positive culture was included in each series of tests. For β -D-glucosidase tests, the positive control culture was *Acholeplasma laidlawii* PG8 and for phosphatase it was *Mycoplasma arthritidis* PG6.

A 10- μ l drop of 4MU substrate in buffer was added to each test area including controls, and the organisms were mixed with the substrate. Disposable plastic tips mounted on a 10- μ l micropipette (Eppendorf, Hamburg, West Germany) were suitable for this as they did not scratch the glass.

The plate was placed in a humid chamber and incubated at 37°C for 60 min. A 20- μ l drop of sodium glycine buffer, pH 10.4 (0.2 M), was then added to each test area and the plate was gently rocked to aid mixing. This high pH gives an increase in the fluorescent intensity of 4MU (5).

The plate was viewed in a dark room against a black background under an ultraviolet lamp emitting light at 365 nm, and the test areas were examined for fluorescence relative to the neighboring controls (Fig. 3).

Comparison of fluorogenic and conventional

FIG. 1. Procedure for fluorogenic test.

FIG. 2. *Mycoplasma* organisms on glass plate (Diameter of circle = 10 mm).

tests for β -D-glucosidase and phosphatase. The procedures used were the same for both enzymes and Tables 1, 2, and 3 show which species were examined. Cultures were grown on routine agar medium for 48 h, and colonies were then transferred to a glass plate for the rapid fluorogenic test with the appropriate 4MU substrate. Colonies from the same agar plate were also transferred by "push-block" onto the corresponding conventional test medium. Control uninoculated plates were included, and these conventional tests were read after 3, 7, and 14 days of incubation according to standard methods (1, 9).

Each test culture was examined on at least two separate occasions.

RESULTS

The fluorogenic and conventional methods gave similar results for β -D-glucosidase activity as shown in Table 1, and it was noted that the intensity of reaction also correlated in most tests.

Phosphatase activity of the *Mycoplasma* species was equally well detected by both tests (Table 2), but the presence of Mg^{2+} ions noticeably enhanced the fluorogenic reactions. Phos-

FIG. 3. Example of fluorogenic test under illumination at 365 nm. Left hand side, negative control; Right hand side, positive test.

TABLE 3. Tests for phosphatase activity in *A. laidlawii* strains

Strain	Conventional test ^a	Fluorogenic test ^a	
		With Mg^{2+}	Without Mg^{2+}
PG8	+ ^b	+ ^b	-/w+ ^b
PG9	w+/+	w+/+	-
S-Algen	w+/+	w+/+	-
T91/L31	+	+	+
S-Mist	w+/+	w+/+	-
BJ29	+	w+/+	-
C881	+	+	+
C884	+	+	+
M996/75	w+/+	+	+
M1326/75	+	+	w+/+
M2332/75	+	+	-/w+

^a Symbols: +, clear positive reaction; w+, weak positive reaction; -, negative reaction.

^b Each strain was tested on two separate occasions but two results are given only where they were not consistent.

phatase activity was detected in all the *A. laidlawii* strains by both tests, although Mg^{2+} ions were essential for the fluorogenic test, some cultures being negative in their absence (Table 3).

Several strains of this organism reacted inconsistently, giving a clear-cut positive reaction

in one test and only a weak reaction in the other, but this applied to both the conventional and the fluorogenic test.

DISCUSSION

These results suggest that the fluorogenic tests as described for β -D-glucosidase and for phosphatase are as accurate as their conventional counterparts and have the advantage that a considerable amount of time is saved. It may be possible to complete the fluorogenic tests in even less than 1 h, although pilot studies on a limited number of mycoplasma species suggested that this was a suitable period of incubation. These pilot tests also indicated that the use of 48-h cultures was not critical and that 72-h cultures were as suitable for these two particular enzymes. For fast-growing species it may well be possible to use younger cultures.

In initial experiments additional controls were included to confirm that the generation of fluorescence was enzymatic in nature. Thus, it was shown that fluorescence was not produced by organisms in the absence of substrate and that activity in the presence of substrate was abolished after treatment of colony impressions with either boiling water for 10 min or with 5% trichloroacetic acid for 2 to 3 min (unpublished observations).

Phosphatase reactions were noticeably enhanced in the presence of Mg^{2+} ions, which presumably activated the enzyme. The effect of adding Mg^{2+} ions to conventional phosphatase medium was not investigated, but it is possible that the requirement is already met by the serum component in the medium.

Phosphatase activity was also pH dependent, and it was noted in pilot experiments that acid pH (4.8) or alkaline pH (10.5) were preferred to near-neutral pH, as some reactions were not detectable at pH 7.5. Reactions in acid pH were marginally better than alkaline pH; however, a fuller investigation of the pH optimum of phosphatase would be of value.

Other 4MU substrates currently under examination include a range of fatty acid esters for detecting lipolytic activity of mycoplasmas. It is hoped that they will provide more standardized conditions for this than the currently used "films and spots" test shows, in which the activity has been shown to vary with the medium composition (4).

Apart from offering more standardized substrates than conventional media, and a saving in time, a further advantage of the fluorogenic tests is that the organisms are grown before addition of the substrate so that the problem of growth inhibition by the substrate does not occur. For example, growth of *Mycoplasma dis-*

par was reported to be inhibited in esculin medium, and β -D-glucosidase activity could therefore not be determined (3). In addition, although it has not been investigated, it is probable that the substrates are suitable for quantitative enzymatic assays on the *Mycoplasma*-*tales*.

Since the completion of this work the author's attention has been drawn to a somewhat similar procedure for detecting enzymes in bacterial cultures (8). The tests, which utilized a range of 4MU substrates for glycosidase enzymes, were conducted on filter papers after the transfer of bacterial colonies with a glass rod. However, our method using a rubber stopper and glass plate offers certain advantages, particularly for the study of mycoplasmas, whose colony growth is generally not as luxuriant as that of bacteria. The rubber stopper permits a larger sample of culture to be transferred for testing and, unlike the filter paper, the glass plate can be inspected under a microscope to ensure that adequate material has been taken. A rubber stopper was found to be valuable when transferring bacteria instead of a velvet replicator (7), which suggests that it would be suitable for bacteria in this test also. Furthermore, the assessment of weak reactions is also facilitated in our method by using a dark background and by the fact that the reactants remain in a discrete drop on the glass.

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