The Growth-inhibitory Effects of Some Dyes on Different *Mycoplasma* and *Acholeplasma* spp.

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(Received 29 August 1984)

A microtitre-plate method for evaluating the inhibitory effect of dyes on the growth of mycoplasmas in fluid medium is described. Different species were shown to differ in their sensitivity to dyes. Statistical analysis (a) compared the general sensitivity and resistance of different mycoplasma species to the dyes and (b) showed that the dyes fell into two main groups in their effects on the mycoplasma species.

INTRODUCTION

Although dye sensitivity has been used to distinguish between species of bacteria such as *Brucella* spp. which vary in their reaction to basic fuchsin and thionin (Mackie & McCartney, 1956) very little is known about the inhibitory effect of most dyes on the growth of *Mycoplasma* and *Acholeplasma* spp. Methylene blue, which has differential inhibitory effects on human mycoplasmas (Kraybill & Crawford, 1965), is used in a medium for selectively isolating *Mycoplasma pneumoniae* (Smith *et al.*, 1967), at concentrations that inhibit other *Mycoplasma* spp. of the human respiratory tract.

This paper reports the sensitivity of a large number of mycoplasma species and strains to various dyes. The aims included assessment of variability between and within species and whether other dyes besides methylene blue might be of value in the recovery of specific species from mixed infections.

METHODS

Mycoplasma and *Acholeplasma* spp. Mycoplasma type cultures (Table 1) were obtained from the National Collection of Type Cultures, London NW9, UK and Drs M. F. Barile (National Institutes of Health, Bethesda, Md., USA), R. J. Fallon (Ruchill Hospital, Glasgow, UK), J. T. Heywood (National Communicable Disease Centre, Atlanta, Ga., USA), H. Kirchoff (Tierärztliche Hochschule, Hannover, FRG), R. H. Leach (PHLS Mycoplasma Reference Laboratory, Norwich, UK), G. Smith (Zoological Society of London, London, UK) and D. Taylor-Robinson (Clinical Research Centre, Harrow, UK). Some unidentified primate mycoplasmas were received from Drs B. C. Cole (University of Utah, Utah, USA) and A. Polak-Vogelzang, (Rijks Instituut voor de Volksgezondheid, Bilthoven, Netherlands) and others were isolated at the former MRC Laboratory Animals Centre from animals sent for diagnosis of disease or for microbiological screening. Four strains of *Mycoplasma cricetuli* and three strains of *M. collis* were examined. Some serological cross-reaction has been shown between *M. felis* and the feline strains LA, LC, LY and LP (Hill, 1975); between *M. faucium* and the primate strains 2IE, HSPP, SPF and 26D (Somerson & Cole, 1979, p. 210); and among the eleven guinea-pig strains (Table 1).

Medium and growth conditions. The basic culture medium was that described by Taylor-Robinson *et al.* (1968). Depending upon their biochemical activity mycoplasmas were grown in basic liquid medium containing either 1% (w/v) glucose, pH 7.8 or 1% (w/v) arginine, pH 7.3. Aliquots of cultures were stored in sealed ampoules at –70 °C.

Dyes. The dyes tested are shown in Table 1. Dyes were dissolved in distilled water except for basic fuchsin which was first dissolved in ethanol and then diluted in distilled water. For the dye sensitivity tests, these stock solutions were further diluted in growth medium, as indicated below, to give a range of final concentrations between 0.5% and 0.000005%.

Fig. 1. Agar plate seeded with 12 drops (1-12) of mycoplasma suspension from wells of one row of the microtitre plate, with a gradual decrease from the highest (1) to the lowest (12) concentrations of dye. C. Drop of mycoplasma suspension from a control well lacking dye.

Mycoplasma viability titres. Ampoules of liquid cultures were thawed and diluted tenfold to 10^{-6} . Each dilution was then further diluted 1 in 4, in a microtitre plate, in liquid medium containing glucose or arginine as appropriate and with 0.002% phenol red. The plate was sealed and incubated at 35 °C for 3 to 5 d. The mycoplasma viability titre was the dilution with the lowest number of organisms which produced a distinct pH change. Another ampoule from the titrated batch was diluted to the required concentration of organisms for use in the subsequent dye-sensitivity test. The concentration of mycoplasmas used in this test was based upon their ability to effect a pH change within a certain time as a comparison showed little variation in growth inhibition reactions when using different concentrations of organisms. Strain GP3 and related strains, which metabolized neither glucose nor arginine (Cassell & Hill, 1979, pp. 238 & 262), were tested in medium containing 0.004% (w/v) resazurin, which was reduced during growth producing a colour change from blue to purple-red.

Sensitivity to dyes. A drop (0.05 ml) of the appropriate medium (without phenol red) was deposited in each of a row of wells in a microtitre plate. The stock solution of the dye under test (0.05 ml) was added to the first well and doubling dilutions were made to well 12. Medium (0.1 ml) was added to each well, followed by 0.05 ml of the mycoplasma suspension at a dilution calculated to produce a pH change in 3 to 5 d in control medium wells containing phenol red but no dye. Plates were sealed and incubated at 35 °C. When the control well showed a pH change of half a unit (or a distinct colour change in medium containing resazurin) viability was examined by inoculation of a standard drop (0.025 ml) of suspension taken from each well, including controls, onto an agar plate (visual estimation of growth, as in conventional metabolism tests, was precluded by the presence of dyes). Plates were incubated and examined for growth from each well. Absence of growth from tests wells indicated growth inhibition, provided control wells were positive; the inhibition titre was the highest dilution of dye causing growth inhibition (Fig. 1).

Inhibition titres determined by this method were compared with those obtained by the more conventional method of Hill & Sutton (1979) based upon serological metabolism tests (Purcell *et al.*, 1966; Taylor-Robinson *et al.*, 1966). For these tests the colourless antibacterials, potassium tellurite and sodium desoxycholate, were used in media with phenol red. Results were recorded by reading the titres based on (a) pH change and (b) colonial growth on seeded plates as above.

Statistical analysis. The log of the dye inhibition titre was subjected to three types of statistical analysis.

(1) Single linkage cluster analysis (Hartigan, 1979) was used to show the explicit relationships among the dyes with respect to their effects on mycoplasmas (Dunn & Everitt, 1982). For example, two dyes which affected all species similarly would be closely related, while two in which the patterns of sensitivity among species differed would be less closely related or unrelated. The technique starts by finding the two dyes (or mycoplasmas, see (2) below) which have the greatest similarity, and these become the first cluster. The similarities between this cluster and each of the remaining dyes is then calculated, and the closest one is added to the first cluster, and so on until

all dyes form a single cluster. The pattern of agglomeration can be represented by a dendrogram, representing the similarities between the dyes.

(2) Single linkage cluster analysis was also used to study the similarity of the mycoplasmas as judged by the pattern of dye sensitivity. The resulting dendrogram was too large to be published, but served to highlight those species which behaved similarly in their responses to the dyes. These are noted in the results section.

Although this second analysis shows similarities between mycoplasmas, it cannot indicate the nature of these similarities. Thus, two species may be similar because they are both very sensitive to all dyes (for example) or because they are both sensitive to one dye and resistant to the rest. The nature of the similarities between species can be explored to some extent by principal components analysis.

(3) Principal components analysis (Afifi & Azen, 1979) was used to study in more detail the pattern of response among mycoplasmas to the dyes. Very briefly, principal components analysis is a method for reducing the dimensionality of a set of variables by constructing uncorrelated linear combinations of them which may have some biological meaning. For example, in this study the response of an individual mycoplasma species could be represented by a point in seven-dimensional space. Thus its response to safranin and methylene blue (for example) could be shown on a two-dimensional graph, its response to thionine could be shown by adding a third dimension, its response to resazurin by adding a fourth dimension, etc. Unfortunately, it is impossible to interpret relationships in seven dimensions. Principal components analysis depends on the patterns of correlations among the variables to reduce the dimensionality and produce a new set of variables which may be interpretable. This is clarified in the results sections.

RESULTS AND DISCUSSION

The sensitivity of the mycoplasmas to the dyes is shown in Table 1. Marked variations in sensitivity were shown by mycoplasmas to all the dyes tested. The general ranges of mycoplasma sensitivity to the dyes were as follows: Acriflavine (24 μg to 12 mg litre⁻¹), basic fuchsin (0.6 mg to 0.3 g litre⁻¹), crystal violet (61 μg to 16 mg litre⁻¹), methylene blue (61 μg to 0.1 g litre⁻¹), resazurin (12 mg to 1.2 g litre⁻¹), safranin (0.6 mg to 7.8 mg litre⁻¹), thionin (61 μg to 0.1 g litre⁻¹). Growth inhibition of the six mycoplasmas, *M. bovis genitalium*, *M. caviae*, *M. gallinarum*, *M. neurolyticum*, *Acholeplasma axanthum* and *A. oculi*, compared using the dye sensitivity technique described above and the more conventional visual method of recording a pH change, showed a range of sensitivities to potassium tellurite (0.02 to 5 mg litre⁻¹) and sodium desoxycholate (2 to 50 mg litre⁻¹).

The modified metabolism inhibition (MI) test used to examine the sensitivity of mycoplasmas to antibiotics (Hill & Sutton, 1979) is useful for estimating the sensitivity to colourless chemicals, but cannot be used for testing dyes. The present adaptation was developed, therefore, as an inhibition method for testing dyes, and it proved to be easily-performed. Furthermore the titres recorded by this method were comparable with those obtained by the more conventional MI-type test, when the sensitivity to colourless chemicals was examined.

The sensitivity of *M. pneumoniae* to methylene blue was very much lower than for all other primate mycoplasmas, confirming the observations of Kraybill & Crawford (1965), and supporting its use in medium for isolating *M. pneumoniae* from mixed infections. The reactions shown in Table 1 suggest that thionin, incorporated in the medium, might be of value in recovering *M. collis* from mixed rodent infections as *M. collis* is less sensitive than *M. pulmonis* to this dye. Crystal violet was the only dye tested which might distinguish the two common human mycoplasmas, *M. hominis* and *M. salivarium*.

The result of the cluster analysis, showing the relationships between the dyes, is shown in the dendrogram, Fig. 2. This shows that the dyes fall into two main clusters, with methylene blue, resazurin and thionin behaving rather similarly in one and acriflavine, basic fuchsin, safranin and crystal violet in the other. Thus, if a restricted set of dyes were to be used in characterizing species it would be better to use ones that are as distantly related as possible in their patterns of inhibition such as basic fuchsin and methylene blue.

The principal components analysis was successful in reducing the seven dimensions of the raw data (one dimension for each dye) to only two significant new dimensions which together accounted for 68% of the total variation. The first of these components, which accounted for 43% of the total variation, was correlated about equally with all the dyes and can be interpreted

Table 1. *Sensitivity of mycoplasmas to dyes*

Values are $\log_{10}$ of the lowest dye concentration ($\mu\text{g litre}^{-1}$) at which the organisms failed to grow. Braces denote strains belonging to the same species.

Mycoplasmas	Dye						
	Acriflavine	Basic fuchsin	Crystal violet	Methylene blue	Resazurin	Safranin	Thionin
<i>Mycoplasma agalactiae</i>	4.10	4.89	3.59	4.80	5.49	4.59	4.49
<i>M. alkalescens</i>	3.49	4.89	3.89	5.10	5.49	4.29	4.49
<i>M. alvi</i>	2.89	4.59	3.89	1.79	3.09	3.39	2.39
<i>M. anatis</i>	1.99	4.89	3.89	2.39	5.49	3.99	4.49
<i>M. arginini</i>	3.80	5.19	3.89	3.89	5.19	4.59	4.19
<i>M. arthritis</i>	3.19	4.89	3.59	3.89	5.19	4.29	3.59
<i>Acholeplasma axanthum</i>	3.49	4.89	3.89	4.49	4.29	4.59	4.80
<i>M. bovirhinitis</i>	3.80	4.59	3.59	4.19	5.80	4.29	4.80
<i>M. bovirhinis</i>	3.80	5.19	4.19	4.80	6.10	4.29	5.10
<i>M. bovis</i>	3.80	4.89	3.29	2.39	4.89	4.89	2.99
<i>M. bovoculi</i>	2.89	3.99	2.99	3.89	4.59	3.69	2.09
<i>M. buccale</i>	2.59	3.99	2.99	2.69	4.89	4.29	2.09
<i>M. canadense</i>	3.19	4.59	3.29	3.59	4.59	3.69	3.89
<i>M. canis</i>	3.49	4.89	3.29	4.19	6.10	4.29	4.49
<i>M. capricolum</i>	3.49	5.19	4.19	3.29	4.29	4.89	2.09
<i>M. caviae</i>	3.49	4.89	2.99	2.09	3.39	3.99	3.29
<i>M. citelli</i>	3.19	4.89	2.39	2.39	3.69	3.39	2.39
<i>Spiroplasma citri</i>	2.89	4.29	3.89	4.19	5.19	3.08	3.89
<i>M. columbinum</i>	4.10	5.19	4.19	> 5.10	5.49	4.59	4.80
<i>M. columborale</i>	3.49	4.59	3.29	3.89	4.89	3.69	4.19
<i>M. conjunctivae</i>	1.39	3.99	2.69	3.90	3.69	3.69	2.99
<i>M. cynos</i>	2.89	4.59	3.59	2.99	5.19	3.69	3.89
<i>M. dispar</i>	3.49	4.59	2.09	3.89	5.80	3.99	3.29
<i>M. edwardii</i>	3.49	4.29	2.39	5.10	4.89	3.99	3.89
<i>A. equifetale</i>	3.19	4.59	3.89	4.19	4.89	3.99	4.80
<i>M. equigenitalium</i>	3.49	4.59	2.09	2.09	5.19	3.69	4.19
<i>M. equirhinis</i>	3.49	4.59	2.99	< 1.79	4.29	3.99	2.99
<i>M. fastidiosum</i>	2.29	3.39	< 1.79	< 1.79	4.29	< 2.79	< 1.79
<i>M. faucium</i>	3.80	4.59	< 1.79	< 1.79	3.99	3.39	< 1.79
<i>M. feliminutum</i>	2.89	3.99	3.89	< 1.79	3.09	< 2.79	< 1.79
<i>M. felis</i>	3.49	4.59	< 1.79	3.59	5.19	3.99	4.19
<i>M. fermentans</i>	3.49	4.59	2.99	2.99	4.89	3.39	3.89
<i>M. gallinarum</i>	4.10	5.19	2.99	4.49	5.80	4.29	4.49
<i>M. gallisepticum</i>	2.59	4.29	3.29	4.49	4.59	4.29	2.99
<i>M. gateae</i>	3.80	4.89	2.69	2.69	4.29	3.69	2.99
<i>A. granularum</i>	2.59	3.39	4.19	4.49	4.89	4.59	4.49
<i>A. hippikon</i>	3.19	3.99	3.89	4.49	5.19	3.99	4.49
<i>M. hominis</i>	3.19	4.59	< 1.79	< 1.79	4.59	3.39	2.99
<i>M. hyorhinis</i>	2.29	4.59	3.29	4.49	4.59	3.39	3.59
<i>M. iners</i>	> 4.10	5.19	3.89	2.69	6.10	4.59	3.89
<i>A. laidlawii</i>	2.89	4.89	3.89	4.49	5.19	3.99	4.80
<i>M. lipophilum</i>	3.49	4.89	4.19	2.99	5.49	4.29	3.89
<i>M. maculosum</i>	3.80	5.19	3.29	4.19	4.89	4.29	4.19
<i>M. meleagridis</i>	3.80	4.29	2.99	3.59	4.89	4.59	4.19
<i>M. moatsii</i>	2.29	5.19	2.69	2.99	4.89	4.29	3.29
<i>A. modicum</i>	3.19	4.29	3.59	4.49	5.19	4.29	4.80
<i>M. molare</i>	2.29	3.99	3.29	3.89	4.59	3.39	3.29
<i>M. mycoides</i>							
subsp. <i>capri</i>	4.10	5.49	3.89	3.29	4.59	4.89	2.09
<i>M. mycoides</i>							
subsp. <i>mycoides</i>	2.89	4.59	2.69	2.39	4.29	3.99	2.69
<i>M. neurolyticum</i>	1.99	4.29	3.29	2.09	3.39	< 2.79	3.29
<i>A. oculi</i>	2.29	3.69	3.89	4.80	4.89	3.99	4.19
<i>M. opalescens</i>	3.19	4.59	3.89	2.99	5.80	3.09	4.19
<i>M. orale</i>	3.49	4.29	3.29	2.39	4.29	4.59	2.99
<i>M. pneumoniae</i>	2.29	4.29	< 1.79	> 5.10	5.19	3.69	4.19
<i>M. primum</i>	3.80	4.89	3.59	3.59	5.19	3.99	4.19

Table 1 (continued)

Mycoplasmas	Dye						
	Acriflavine	Basic fuchsin	Crystal violet	Methylene blue	Resazurin	Safranin	Thionin
<i>M. pulmonis</i>	3.49	4.29	2.69	< 1.79	4.29	< 2.79	< 1.79
<i>M. putrefaciens</i>	3.80	4.89	2.69	3.29	4.29	4.29	2.39
<i>M. salivarium</i>	3.49	4.89	3.59	< 1.79	3.99	4.29	3.29
<i>M. spumans</i>	3.80	4.59	3.29	3.89	5.19	4.89	4.19
<i>M. sualvi</i>	3.19	5.49	2.69	3.59	4.59	4.89	3.29
<i>M. subdolum</i>	3.49	4.89	2.99	< 1.79	5.19	4.29	4.19
<i>M. verecundum</i>	3.80	4.89	2.99	4.49	6.10	4.29	4.80
<i>M. cricetuli</i> strains:							
{ CH	2.89	3.99	< 1.79	3.29	4.29	< 2.79	2.09
{ SH	2.89	4.29	< 1.79	2.09	3.69	< 2.79	2.09
{ LE	3.19	4.29	< 1.79	2.39	4.29	< 2.79	2.69
{ HO	3.19	3.99	< 1.79	< 1.79	4.29	< 2.79	2.09
<i>M. collis</i> strains:							
{ 58B	1.99	4.59	< 1.79	2.99	4.59	3.69	3.29
{ GOUGH	2.89	4.59	< 1.79	3.29	4.59	3.39	3.29
{ LIL	2.29	4.59	2.69	3.29	4.59	3.69	3.59
Guinea-pig							
<i>Acholeplasma</i> strains:							
{ GP3	2.89	< 2.79	2.39	2.69	5.19	3.09	4.19
{ 22A	2.89	3.99	3.29	4.49	4.89	3.69	4.49
{ 158G	2.59	3.39	2.39	2.99	5.49	3.39	4.80
{ 3VU	3.49	4.29	3.59	4.19	5.80	3.99	4.49
{ 148J	2.59	< 2.79	2.39	3.59	5.49	< 2.79	4.19
{ 12K	2.59	3.39	2.69	4.19	5.49	4.60	4.49
{ 23K	2.89	3.39	2.29	4.19	5.49	4.29	4.19
{ 75K	2.59	< 2.79	2.39	4.19	5.49	< 2.79	4.19
{ 83K	2.59	< 2.79	2.69	4.19	5.49	< 2.79	4.49
{ 118K	3.19	3.39	3.29	4.19	5.80	4.59	4.49
{ 128K	3.19	4.29	2.69	3.59	5.80	< 2.79	4.49
Feline strains							
(<i>M. felis</i> -related?):							
LA	3.80	4.59	2.39	3.59	4.29	3.39	3.59
LC	3.49	4.29	2.99	3.89	5.19	3.99	4.49
LY	2.29	4.59	< 1.79	< 1.79	4.29	3.69	2.99
LP	1.69	4.89	3.59	3.59	4.89	3.69	3.89
Primate strains							
(<i>M. faucium</i> -related?):							
21E	2.89	4.59	2.99	2.39	4.89	4.29	4.19
HSPP	3.49	4.89	2.69	< 1.79	4.29	3.69	2.39
SPF	3.80	4.89	4.19	2.39	3.99	4.29	2.39
26D	3.80	4.59	2.69	2.69	3.99	3.99	3.89

as a factor representing general sensitivity/resistance to the dyes. In other words, 43% of the total variation was associated with a tendency of the mycoplasma species to be either sensitive or resistant to all the dyes. When the strains were ranked according to their score on this component, the most resistant species were *M. bovis genitalium*, *M. columbinum* and *M. agalactiae*. The most sensitive were *M. neurolyticum*, *M. feliminutum*, *M. cricetuli* (strains HO and SH) and *M. fastidiosum*.

The second principal component accounted for 25% of the variation and was positively correlated with response to acriflavine, basic fuchsin, crystal violet and safranin and was

Fig. 2. Dendrogram showing similarities between dyes in their effect on the mycoplasmas.

negatively correlated with the other three dyes. This component represents differential sensitivity of the mycoplasmas to the two main groups of dyes shown in the dendrogram, Fig. 2. When ranked according to their scores on this component all the guinea-pig *Acholeplasma* strains proved to be relatively sensitive to the methylene blue group of dyes and resistant to the acriflavin group. In contrast, *M. caviae*, *M. salivarium*, strain SPF, *M. capricolum* and *M. mycoides* subsp. *capri* were relatively resistant to the methylene blue and sensitive to the acriflavine groups of dyes.

The overall sensitivity of the mycoplasmas to the dyes may be associated with some general property such as permeability of the cell membrane, while specific patterns of response to individual dyes may be associated with differences in metabolic detoxification mechanisms between species.

I would like to thank Dr M. Festing for carrying out computer analysis of the data and for his advice, and Mr I. Whisson for the photographic print.

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