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Antibiotic sensitivity and mutation rates to antibiotic resistance in *Mycoplasma mycoides* ssp. *mycoides*

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SUMMARY

The antibiotic resistance of *Mycoplasma mycoides* ssp. *mycoides* strain T_1 was investigated. This strain was resistant to high levels ($> 100\ \mu g\ ml^{-1}$) of rifampicin and nalidixic acid. It was sensitive to streptomycin, spectinomycin and novobiocin; however, single step mutants with high levels of resistance ($> 100\ \mu g\ ml^{-1}$) were readily isolated. With erythromycin and tylosin for which the minimum inhibitory concentration (MIC) for the parent strain was $< 0{\cdot}1\ \mu g\ ml^{-1}$, mutants resistant to $> 100\ \mu g\ ml^{-1}$ were obtained in two and three steps respectively. The MIC of tetracycline in single step resistant mutants ($0{\cdot}6\ \mu g\ ml^{-1}$) was tenfold higher than the parent strain, but could not be increased further. There was only a twofold increase in resistance to chloramphenicol in single step mutants. The frequency of resistant mutants varied with the antibiotic and was between 4×10^{-6} and 2×10^{-8}. The mutation rate to antibiotic resistance to streptomycin, spectinomycin, novobiocin, erythromycin and tylosin was between 3×10^{-8} and 5×10^{-9} per cell per generation. There was a fivefold decrease in mutation rate to resistance to 60 $\mu g\ ml^{-1}$ streptomycin compared to that to 20 $\mu g\ ml^{-1}$.

INTRODUCTION

There is relatively little information in the literature concerning antibiotic resistance in mycoplasmas. In particular, there are no reports on mutation rates, and few on frequencies of resistance in cell populations.

In the work reported in this paper the antibiotic resistance of *Mycoplasma mycoides* ssp. *mycoides*, the causative agent of contagious bovine pleuropneumonia (CBPP), was investigated. Minimum inhibitory concentration (MIC) values for strain T_1 and for antibiotic-resistant strains derived from it, and the frequencies of resistant organisms were determined; mutation rates to resistance to some antibiotics were also determined using a fluctuation test (Luria & Delbrük, 1943). In addition to providing information on antibiotic resistance, it was envisaged that the study might indicate the potential usefulness of antibiotic resistant markers in genetic studies. We have previously reported the isolation of substrate-negative mutants of *M. mycoides* (Lee, Miles & Beezer, 1986).

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MATERIALS AND METHODS

Organism, media and preparation of inocula

Mycoplasma mycoides ssp. *mycoides* strain T_1 was grown in a glucose-tryptose-serum broth and stored in liquid nitrogen as described previously (Lee, Miles & Perry, 1985). Cells recovered from liquid nitrogen were inoculated into tubes of broth (200 μl per 10 ml broth) and incubated statically at 37 °C for 48 h to provide inocula for experiments. Viable counts were also performed as previously described using a surface drop-plate method. Where appropriate, cell suspensions were diluted in $\frac{1}{4}$ strength Ringer solution (pH 7·2).

Antibiotics

Streptomycin, spectinomycin, novobiocin and tylosin were prepared in distilled water, chloramphenicol and erythromycin in ethanol, tetracycline in methanol, nalidixic acid in 1 M-NaOH and rifampicin in dimethylsulphoxide (DMSO); the solvents used were as suggested by Washington & Barry (1974). Stock solutions of antibiotic other than rifampicin were filter sterilized (Millipore filter, pore-size 0·22 μm), distributed in 2 ml ampoules and stored at −20 °C; rifampicin in DMSO was used without sterilization, but was similarly stored. In control experiments, viable counts of cell suspensions were not significantly affected by the inclusion in glucose-tryptose-serum agar of the small amounts of NaOH, ethanol, methanol or DMSO used to initially solubilize antibiotics.

Determination of the MIC and the frequency of antibiotic-resistant mutants

The agar dilution method recommended by Ericsson & Sherris (1971) was used. Antibiotic solutions were prepared in a twofold dilution series; however, to allow accurate determination of MIC values, additional dilutions giving final concentrations of antibiotic in agar medium of 0·4, 0·6, 0·8, 1·0, 1·2 and 1·4 times preliminary estimates of the MIC were also used.

Antibiotic was added to bottles of molten agar at 50 °C. Bottles were then inverted five times to ensure thorough mixing (Snyder *et al.* 1976) and poured to a depth of approximately 3 mm. At each antibiotic concentration two or more plates were inoculated with 80 μl of a 48 h broth culture (viable count approximately 2×10^9 c.f.u. ml^{-1}) diluted, as appropriate, in $\frac{1}{4}$ strength Ringer solution. After drying, plates were incubated in air, in sealed containers at 37 °C. Plates containing streptomycin were also incubated in an anaerobe jar using a gas generator giving 90% hydrogen and 10% carbon dioxide (Gas Pak, Oxoid). Viable counts were determined at 7 days. The lowest antibiotic concentration which prevented growth with an inoculum of 10^5 c.f.u. per plate was regarded as the MIC, except that growth with $\leqslant 2$ colonies per plate was disregarded.

The frequency of mutants capable of growth at antibiotic concentrations $\geqslant$ MIC was determined from plates inoculated with 10^6, 10^7 or 10^8 c.f.u.; the mean number of colonies appearing on plates was divided by the number of c.f.u. inoculated.

Determination of the mutation rate to antibiotic resistance

The mutation rate was determined by a fluctuation test similar to that used by Savva (1980) with various strains of Enterobacteriaceae.

Broth medium without glucose was inoculated with a 48 h culture, which had been stored in liquid-nitrogen, to give a final organism concentration of 10^4 c.f.u. ml^{-1}. The suspension was gently stirred with a magnetic rod and dispensed (2 ml aliquots) into the wells of plastic repli dishes (Sterilin, UK; 25 wells per dish); the use of this technique enabled large numbers of replicate cultures to be set up. The viable count and the number of antibiotic resistant mutants at the time of inoculation were determined. In all the experiments reported the numbers of mutants was less than one in 5×10^6 cells. Thus, the probability of any well having a mutant cell at time 0 h was low.

The repli dishes were incubated for 24 h, after which time the viable count of representative wells was again determined; in the medium used (i.e. broth without added glucose) this was approximately 2×10^7 c.f.u. ml^{-1}, and was limited by the concentration of utilizable saccharides in the serum component of the medium. Aliquots (1 ml) of broth medium containing glucose (15 mg ml^{-1}) and the test-antibiotic were then added to each well. The repli dishes were sealed with wax tape to prevent any moisture loss and re-incubated. At the time of antibiotic addition pH had not changed from the initial value of 7·6. Growth after the addition of antibiotic occurred in wells where resistant mutants had arisen prior to antibiotic addition and was detected by a fall in pH. After 12 days incubation four drops of a mixed pH indicator (bromothymol blue 0·5 g, neutral red 0·5 g, ethanol 1 l) were added to each well; the colour change of this indicator is green (alkali) to red (acid) at pH 7·2. Where growth occurred after antibiotic addition the pH fell to approximately 6·6 and the viable count was approximately 10^9 c.f.u. ml^{-1}.

The mutation rate per cell per generation (a), and its standard error, was estimated according to the equation

$$a = \left(x \pm \sqrt{\frac{e^x - 1}{c}}\right) \frac{\ln 2}{(n - n_0)},$$

where x is $-\ln$ (proportion of wells without mutants), n_0 and n are the mean viable counts at 0 and 24 h, and c is the total number of positive and negative wells (Luria & Delbrück, 1943; Lea & Coulson, 1949).

A minimum of five repli dishes were used for each determination of a mutation rate. In each dish there were two control wells, one uninoculated and the other inoculated but without antibiotic addition at 24 h. The viable counts were the mean values from five wells; the 95% confidence limits of the counts were within $\pm 4\%$. The presence of antibiotic resistant mutants in all positive wells (i.e. those in which growth occurred after antibiotic addition) was confirmed by subculture onto agar containing antibiotic.

RESULTS

MICs of antibiotics for M. mycoides *strain* T_1 *and for resistant mutants*

The MICs of various antibiotics for *M. mycoides* and mutant frequency to antibiotic resistance are given in Table 1. Mutant frequencies were calculated assuming that all the colonies appearing on plates containing $\geqslant$ MIC of antibiotic were resistant mutants. Confirmation of this was obtained only at the highest

Table 1. *Minimum inhibitory concentrations (MIC) of various antibiotics for* M. mycoides *and the frequency of antibiotic-resistant mutants*

(The frequencies quoted are the mean of two or more replicate experiments; all frequencies of $< 1 \times 10^{-6}$ were determined from plates inoculated (in duplicate) with $\geqslant 10^8$ c.f.u.)

Antibiotic	MIC (μg ml^{-1})	Mutant frequency $\times 10^{-8}$ at 1 × MIC	2 × MIC	4 × MIC	10 × MIC	100 × MIC
Streptomycin	10·00	V	58	10	4	5
Streptomycin*	10·00	V	75	16	4	4
Spectinomycin	6·00	V	400	120	40	42
Novobiocin	20·00	10	2	3	NC	ND
Chloramphenicol	1·00	2	2	NC	NC	ND
Erythromycin	0·04	V	84	49	ND	ND
Nalidixic acid	200·00	V	ND	ND	ND	ND
Rifampicin	> 400·00	ND	ND	ND	ND	ND
Tetracycline	0·04	V	450	ND	ND	ND
Tylosin	0·04	3	2	NC	ND	ND

* Determined under anaerobic conditions.
NC, No resistant colonies isolated.
ND, Not determined.
V, Frequency variable and dependent upon inoculum size (see text).

antibiotic concentrations at which growth was observed. Representative colonies were subcultured into broth without antibiotic and after incubation it was shown that in all cases the MIC had increased by at least eightfold, except with chloramphenicol where the MIC was $\leqslant$ twice that of the parent strain.

M. mycoides was resistant to 400 μg ml^{-1} rifampicin and 100 μg ml^{-1} nalidixic acid; furthermore, with the latter antibiotic, although 200 μg ml^{-1} prevented growth with an inoculum of 10^5 c.f.u. per plate, a substantial growth was obtained with an inoculum of 10^6 c.f.u. per plate, indicating a strong dependence of inhibition upon inoculum size. The MIC of the other antibiotics tested ranged from 0·04 to 20 μg ml^{-1}. It can be seen from Table 1 that single step mutants resistant to high levels of streptomycin and spectinomycin were readily obtained; these mutants had MIC values in excess of 2000 μg ml^{-1} antibiotic. Novobiocin resistant mutants also had high MIC values ($\geqslant 800$ μg ml^{-1}).

With erythromycin, tetracycline and tylosin single step mutants did not show a high level of resistance. Colonies growing in the presence of the highest concentration of antibiotic were, therefore, subcultured into broth (without antibiotic), and after incubation, were plated on serum agar plus antibiotic. In this way a single-step mutant for which the MIC of tylosin was 1·6 μg ml^{-1}, gave a further resistant strain with an MIC of 24 μg ml^{-1}; by repeating this procedure once more a strain resistant to > 100 μg ml^{-1} tylosin was obtained. With erythromycin a single step mutant with an MIC of 1·2 μg ml^{-1} gave a further resistant strain (in one step) resistant to > 100 μg ml^{-1}. In ten separate experiments tetracycline resistance could not be increased above that obtained in single step mutants, for which the MIC was 0·6 μg ml^{-1}, i.e. tenfold higher than in the parent strain. Single step mutants resistant to chloramphenicol had MIC values up to twice that of the parent strain, and no attempts were made to isolate multi-step mutants.

The antibiotic-resistance of mutant strains did not diminish on subculture. For each antibiotic, representative resistant mutants were serially passaged in anti-

Table 2. *Mutation rates to antibiotic resistance*

The rates shown are representative values from individual experiments. In replicate experiments (observed mutation rates were always found to fall within the 95% confidence limits.)

Antibiotic	Concentration (μg ml^{-1})	Mutation rate ($\times 10^{-9}$) per cell per generation with 95% confidence limits
Streptomycin	20	$29 \pm 7{\cdot}5$
Streptomycin	60	$6{\cdot}4 \pm 3{\cdot}6$
Spectinomycin	60	$9{\cdot}6 \pm 4{\cdot}8$
Tylosin	0·04	$5{\cdot}9 \pm 3{\cdot}0$
Erythromycin	0·16	$5{\cdot}2 \pm 2{\cdot}8$
Novobiocin	20	$14 \pm 5{\cdot}1$

biotic free broth (100 μl inoculum per 9 ml of broth) for a minimum of three times. In all cases no reduction in antibiotic resistance was observed.

Frequencies of antibiotic-resistant mutants

The frequency with which single-step antibiotic-resistant mutants were isolated from the parent strain at $\geqslant 2 \times$ MIC was between 4×10^{-6} and 2×10^{-8} (Table 1), and multi-step mutants resistant to erythromycin and tylosin were isolated from single step mutants with frequencies of 10^{-7} to 10^{-8}. At $1 \times$ MIC of streptomycin, spectinomycin, nalidixic acid, erythromycin and tetracycline, the frequencies with which colonies appeared depended strongly upon inoculum size, e.g. with streptomycin (10 μg ml^{-1}), an inoculum of 10^7 c.f.u. gave more than 400 colonies whereas an inoculum of 10^6 gave none. These results were reproducible suggesting, possibly, that there is sufficient uptake of antibiotics by large populations to reduce the effective antibiotic concentration below the MIC. This effect was not observed at $\geqslant 2 \times$ MIC where the number of colonies appearing on plates was proportional to the number of c.f.u. added.

Mutation rates to antibiotic resistance

Mutation rates for single-step resistance to streptomycin, spectinomycin, tylosin, erythromycin and novobiocin by the parent strain are given in Table 2. The antibiotic concentration used to assess the presence of mutants was the MIC for novobiocin and tylosin; however, 2–6 times the MIC was used for the other antibiotics, for which growth at the MIC was dependent upon inoculum size.

The mutation rate to resistance to 20 μg ml^{-1} streptomycin was significantly higher than that to 60 μg ml^{-1}. Variation of mutation rate with streptomycin concentration has previously been reported to occur in *Salmonella typhi* and *Pseudomonas aeruginosa*, but not in *Escherichia coli* and *Shigella sp.* (Alexander *et al.* 1950).

DISCUSSION

Of the nine antibiotics studied tylosin, tetracycline and erythromycin were the most active, with MIC values $< 0{\cdot}1$ μg ml^{-1} for *M. mycoides* strain T_1. The MIC values for streptomycin and tylosin (10 and 0·04 μg ml^{-1} respectively) were similar to those previously reported for *M. mycoides* strain V5 (8 and 0·07 μg ml^{-1}; Turner, 1960; Hudson & Etheridge, 1965), though the value for tetracycline was

fourfold lower (Mr G. S. Cottew, footnote to the paper of Turner, 1960). The resistance of *M. mycoides* to rifampicin (400 μg ml^{-1}) is in agreement with reports for other mycoplasmas (Das & Maniloff, 1976).

High level resistance (> 100 μg ml^{-1}) to novobiocin, spectinomycin or streptomycin was achieved in a single step, to erythromycin in two steps and tylosin in three steps. Tetracycline mutants, initially isolated by growth in the presence of 0·08 μg ml^{-1} grew at concentrations of up to 0·6 μg ml^{-1}, but attempts to isolate mutants with resistance greater than 0·6 μg ml^{-1} were unsuccessful. Niitu (1983) has similarly reported that with *M. pneumoniae* resistance may be developed *in vitro* to erythromycin, but not to tetracycline. However, in *M. arginini* (Kenny, 1978) and in bovine strains of mycoplasma (Pilaszek & Truszczynski, 1980b), stepwise increases in resistance to tetracycline have been reported. Also, high MIC values for tetracycline (16 to > 64 μg ml^{-1}) were found in 15% of clinical isolates of *Ureaplasma urealyticum* (Robertson, Coppola & Heisler, 1981) and there are a number of reports of the isolation of tetracycline-resistant strains of *M. hominis* (e.g. Koutsky *et al.* 1983). Tetracycline-resistant strains of *U. urealyticum* and *M. hominis* have recently been shown to contain DNA sequences homologous to the streptococcal tetracycline-resistance determinant *tet* M (Roberts *et al.* 1985; Roberts & Kenny, 1986).

Pilaszek & Truszczynski (1980*a*) determined the MIC of tylosin, an antibiotic recommended for the control of severe reactions to live CBPP vaccine (Hudson, 1971), for 12 bovine strains of mycoplasma. The susceptibility of these strains showed marked variation with MICs ranging from 0·06–60 μg ml^{-1}; those strains with the lowest MIC (0·06 μg ml^{-1}) required the greatest number of passages (16–18) in antibiotic-containing medium to acquire a high level of resistance (100 μg ml^{-1}). Similarly, Hudson & Etheridge (1965) reported that *M. mycoides* strain V5 required ten passages to acquire resistance levels of 100 μg ml^{-1}. These results are in contrast to those presented here in which *M. mycoides*, initially sensitive to 0·04 μg ml^{-1} tylosin, required only three passages to acquire resistance to 100 μg ml^{-1}.

Sensitivity to streptomycin has been reported to be associated with the presence of an electron transport chain, in which oxygen or nitrate acts as the terminal electron acceptor, and which contains functional cytochromes (Bryan, 1982). This association was postulated to reflect a requirement for a functional electron transport chain for streptomycin uptake; the susceptibility of *E. coli* to streptomycin is decreased under anaerobic conditions. Bacteria which do not carry out electron transport to oxygen or nitrate, (e.g. *Clostridium*, *Bacteroides fragilis*) have a very low susceptibility to aminoglycosides (Bryan, Kowand, & Van Den Elzen, 1979). It is of interest, therefore, that *M. mycoides*, which lacks both quinones and cytochromes (Pollack, 1979) was relatively sensitive to streptomycin; this sensitivity was unaffected by incubation in anaerobic conditions (Table 1).

Mutation frequencies of *M. mycoides* to antibiotic resistance (Table 1) range from 4×10^{-6} for tetracycline to 2×10^{-8} for tylosin. There are few previous determinations of mutation frequencies in mycoplasma. Domeruth (1960) reported values of 1×10^{-8} for high level resistance to streptomycin and erythromycin in avian mycoplasmas and Koostra, Adams & Smith (1966) similarly observed values of 10^{-7} to 10^{-9} for resistance to chlortetracycline, chloramphenicol, di-

hydrostreptomycin and erythromycin in a number of mycoplasmas. Kenny (1978) found mutation frequencies to high level streptomycin resistance of 1×10^{-7} for *M. pneumoniae* and *Acholeplasma laidlawii* and 1×10^{-4} for *M. arginini* and *M. hominis*.

Mutation rates to antibiotic resistance in *M. mycoides* were between 3×10^{-8} and 5×10^{-9} per cell per generation. These rates are within the range (2×10^{-7} to 3×10^{-11}) reported for mutation to streptomycin resistance in various Gram negative organisms (Alexander *et al.* 1950), and comparable with the rate for mutation to chloramphenicol resistance (2×10^{-8} to 4×10^{-9}, depending upon cultural conditions) in *Klebsiella aerogenes* (Savva, 1980). The data obtained here do not, therefore, support speculation (see review by Razin, 1985) that there may be a higher mutation rate, and thus a more rapid evolution, in mycoplasmas than in other bacterial groups.

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