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Some *in-vitro* characters of the subspecies of *Mycoplasma mycoides*

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SUMMARY

So-called LC strains of *Mycoplasma mycoides* subsp. *mycoides* and, where appropriate, SC strains, were examined, together with *M. mycoides* subsp. *capri* strains, by growth, fermentation, and antibiotic-sensitivity tests.

Growth curves in BVF-OS medium showed that, from the 6th until at least the 19th day of incubation, strain Mankefår 2833 had a viable count strikingly higher than that of any other LC strain. Its robust growth properties may explain its ability – unusual among LC strains – to produce mycoplasmaemia readily in mice. Strain 143–A66 Conn, also shown by earlier experiments in mice to possess unusual properties, lost viability more rapidly than any other LC strain between the 13th and 19th days of incubation. The viable count of a subsp. *capri* strain was considerably lower than that of any LC strain for much of the period between days 6 and 19.

In fermentation tests with 27 substrates and sensitivity tests with 11 antibiotics the strains gave results that, in all but the following respects, were uniform. Sorbitol was fermented to varying degrees by all the LC and subsp. *capri* strains tested but was unaffected by the SC strains. The LC and subsp. *capri* strains were in general more resistant than the SC strains to streptomycin. The growth of the LC strains, much more than that of the other strains, was greatly stimulated by the presence of fermentable substrate.

INTRODUCTION

Mycoplasma mycoides subsp. *mycoides* is the causative organism of contagious bovine pleuropneumonia (CBPP). Numerous goat and sheep strains of so-called *M. mycoides* subsp. *mycoides* have in recent years been isolated in CBPP-free countries. Such strains are said to be indistinguishable from genuine CBPP strains by the serological tests commonly used in mycoplasmology. A few of them belong to a small colony type and, together with CBPP isolates, are known as 'SC strains'; these strains possess all the characters of genuine *M. mycoides* subsp. *mycoides*. The majority, however, belong to a large colony type and are known as 'LC strains'; in our opinion (Hooker, Smith & Milligan, 1979) the continuing use of the name *M. mycoides* subsp. *mycoides* for the LC strains is misleading.

The LC strains differ from SC strains as follows: they are more proteolytic, and more resistant to a temperature of 45 °C (Cottew & Yeats, 1978); they immunize

mice partially instead of completely against challenge with SC strains (Hooker *et al.* 1979; Smith, Hooker & Milligan, 1980; Smith & Oliphant, 1981*b*); they produce strong – rather than weak – partial protection in mice against challenge with *M. mycoides* subsp. *capri* (Smith & Oliphant, 1981*a*); they are unable – with the single known exception of strain Mankefår 2833 – to produce mycoplasmaemia readily in mice (Hooker *et al.* 1979; Smith *et al.* 1980).

Already there is some evidence that the LC strains do not form an entirely homogeneous group. As stated above, the LC strain Mankefår 2833 is exceptional in that – like the SC strains – it produces mycoplasmaemia readily. Moreover, when Mankefår 2833 is used as a challenge strain in mouse-immunization experiments, vaccines prepared from other LC strains usually give less protection than that provided by homologous-strain vaccine (Smith *et al.* 1980). Cross-immunization experiments have also shown that a second LC strain, 143-A66 Conn, differs in some respects from other LC strains (Smith *et al.* 1980).

This report describes further investigations on the homogeneity of the LC strains and, where appropriate, on their relationship to the SC strains and subsp. *capri*. The features chosen for study were growth curves, fermentation reactions, and sensitivity to antibiotics.

MATERIALS AND METHODS

Mycoplasmas

Strains used to study growth curves, general fermentation reactions, and general antibiotic sensitivity

LC strains of so-called *M. mycoides* subsp. *mycoides* from goats and sheep comprised strains Y goat, Ojo I, Ojo II, Cov 2, 74/2488, 143-A66 Conn, Mankefår 2833, 2605-Razi, Vom/Plum Island, 222-69 N.Y., Ghaleh Morghi-16, S-5-64, and F 30. SC strains comprised P goat and Vom/Parkville from goats, and Blenheim from CBPP. A single strain of *M. mycoides* subsp. *capri* – 'Smith (1423)' – was used. Full details of these strains, including the method of cloning, have already been given (Smith & Oliphant, 1981*a*).

Strains used for definitive tests with sorbitol and streptomycin

The strains were all cloned three times by the filtration method (Report, 1979). They comprised the 17 strains listed above, together with the following: the SC strains Gladysdale, KH_3J, Mara, and Oremit (from CBPP; Smith, 1968), and O goat (Hooker *et al.* 1979); the *M. mycoides* subsp. *capri* strains 74/5907A, JM, YC, and ZZ (isolated in Australia), and BQT (isolated in Turkey), all supplied by Dr G. S. Cottew, C.S.I.R.O., Parkville, Australia.

Measurement of growth curves

The medium used was BVF-OS broth (Turner, Campbell & Dick, 1935) containing Inactivated Calf Serum No. 1 (Wellcome Reagents Ltd). For each strain examined the procedure was as follows. A 10-ml volume of BVF-OS was seeded with 0·1 ml of a 4-day BVF-OS culture and incubated at 37 °C for 24 h, by which time turbidity due to mycoplasmal growth was readily apparent. After a viable count had been made, this 24 h culture was used in a volume of 0·1 ml, or

occasionally more, to seed a further bottle containing 10 ml of BVF-OS. The contents of this bottle were sampled after 3, 6, 10 or 11, 13 or 14, and 19 days' incubation for estimation of the viable count.

The viable-count technique was based on that of Miles, Misra and Irwin (1938). Three 0·02 ml drops of each appropriate dilution in a decimal series were cultured on Blood Agar Base No. 2 with Defibrinated Horse Blood (Oxoid Ltd) 13 %. The colonies were counted after incubation for at least 3 days at 37 °C in a humidified atmosphere.

Fermentation tests

Each fermentation medium was prepared as follows. To 150 ml of 1·0 % Peptone Water (CM9; Oxoid Ltd) was added 2·5 ml of a 0·2 % solution of bromothymol blue (BDH Chemicals Ltd; Cruickshank, 1965). This mixture was autoclaved at 121 °C for 15 min and cooled. With aseptic precautions, 50 ml of sterile Inactivated Calf Serum No. 1 (Wellcome Reagents Ltd) and 22·5 ml of the appropriate 10 % sugar solution (BDH Chemicals Ltd) were added. The final medium, which had a pH of approximately 7·5, was dispensed aseptically in 5-ml volumes in bijou bottles (Sterilin Ltd). It was stored at 4 °C until used.

Each bottle of medium was seeded with one drop of a well-grown 3-day culture in BVF-OS. The tests were read after 1, 3, 6, 10, and 14 days' incubation at 37 °C. A colour change to full yellow was considered a positive result; incomplete colour changes were disregarded. All strains were tested against 27 substrates, and in substrate-free control medium. After 14 days' incubation a loopful from each bottle was cultured on blood agar. Each test included an unseeded series of the 27 fermentation media. Where pH values were measured, a Philips pH meter (PW 9418; Pye–Unicam Ltd, Cambridge) was used.

Sensitivity to antibiotics

General reactions

Tests for sensitivity to 11 agents were made with Multodisks (code nos. U1 and S2; Oxoid Ltd). Each strain was thoroughly streaked on a blood agar plate 15 cm in diameter by means of a swab moistened with a well-grown 3-day culture in BVF-OS. A Multodisk paper was then placed on the surface of the medium. The distance between the edge of each disk and the edge of the zone of growth inhibition was measured after incubation for at least 48 h in a humidified atmosphere at 37 °C.

Definitive test with streptomycin

Blood agar plates containing doubling concentrations of streptomycin (1·22 to 10000 μg/ml) were prepared by adding to each 9-cm Petri dish a mixture containing 8 ml Blood Agar Base No. 2, 1·5 ml defibrinated horse blood, and 0·5 ml aqueous streptomycin sulphate solution (Sigma Chemical Co.). Each plate was inoculated with 4-day BVF-OS cultures of six mycoplasma strains by placing 0·0025-ml drops on the surface of the medium with a calibrated wire loop (Medical Wire and Equipment Co. [Bath] Ltd, Potley, Corsham, Wiltshire). The results, read after incubation for 6 days, were graded '++' (heavy growth), '+' (definite growth, but number and sometimes size of colonies small), and '−' (no growth, but usually slight haemolysis, especially at the periphery of the drop).

RESULTS

Growth curves

Nine LC strains and one subsp. *capri* strain – also a large colony producer – were examined in two similar experiments illustrated in Fig. 1. From the 6th to 19th days of incubation the viable count of strain Mankefår 2833 was strikingly higher than that of any other strain. On day 19 the difference was at least four-fold and usually much greater, but a corresponding difference in optical density did not occur. In both experiments the viable count of Mankefår 2833 rose to $9{\cdot}3 \times 10^9$ or more per ml, and by the 19th day of incubation was still at least $4{\cdot}6 \times 10^9$. The count of the subsp. *capri* strain was considerably lower than that of any other strain for much of the period between days 6 and 19. In both experiments strain 143-A66 Conn showed a striking loss of viability between days 13–14 and 19. In the second experiment all cultures were kept until the 49th day of incubation, by which time all except one (Cov 2) were dead; the Cov 2 culture contained 2×10^6 viable organisms per ml.

Fermentation reactions

General reactions

The following substrates gave negative reactions with all the mycoplasma strains tested (13 LC, 3 SC and 1 subsp. *capri*): adonitol, arabinose, arabitol, cellobiose, dulcitol, erythritol, galactose, glycerol, inositol, inulin, lactose, mannitol, melibiose, raffinose, rhamnose, salicin, sucrose, and xylose. All strains gave positive reactions with starch (in >1 to <3 days), maltose and mannose (>1 to <6 days), dextrin, glucose, glycogen and trehalose (>3 to <10 days), and fructose (>6 to <14 days).

Sorbitol did not give uniform results. Within 14 days eight LC strains (Y goat, 74/2488, 143-A66 Conn, Mankefår 2833, Vom/Plum Island, 222–69 N.Y., Ghaleh Morghi-16, S-5-64) gave positive results (pH 5·35–6·0). The three SC strains and subsp. *capri* remained negative (final pH 7·15—7·25). Five LC strains were also negative in that they failed to produce a complete colour change of the indicator, but their final pH values were 6·4–6·8. All strains were still alive in the sorbitol medium after 14 days' incubation.

The LC strains – but not the SC strains or subsp. *capri* – produced densely turbid growth in the presence of fermentable substrates. The LC strains, in the presence of all fermentable substrates except fructose and sorbitol, were almost always dead by the 14th day of incubation; by contrast the viability of subsp. *capri*, and often that of the SC strains, persisted. All the strains were invariably still alive on the 14th day of incubation in the presence of non-fermentable substrates.

Definitive test with sorbitol

Because sorbitol showed some promise of being of discriminative value, 27 mycoplasma strains (8 SC, 13 LC, and 6 subsp. *capri*) were tested for their ability to ferment it within 14 days.

The final pH values of the eight SC cultures ranged from 7·2 to 7·4 (mean 7·27). Those of the 13 LC cultures ranged from 5·35 to 7·05 (mean 6·27); a colour change to full yellow occurred in 9 of the 13 cultures. The final pH values of the six subsp.

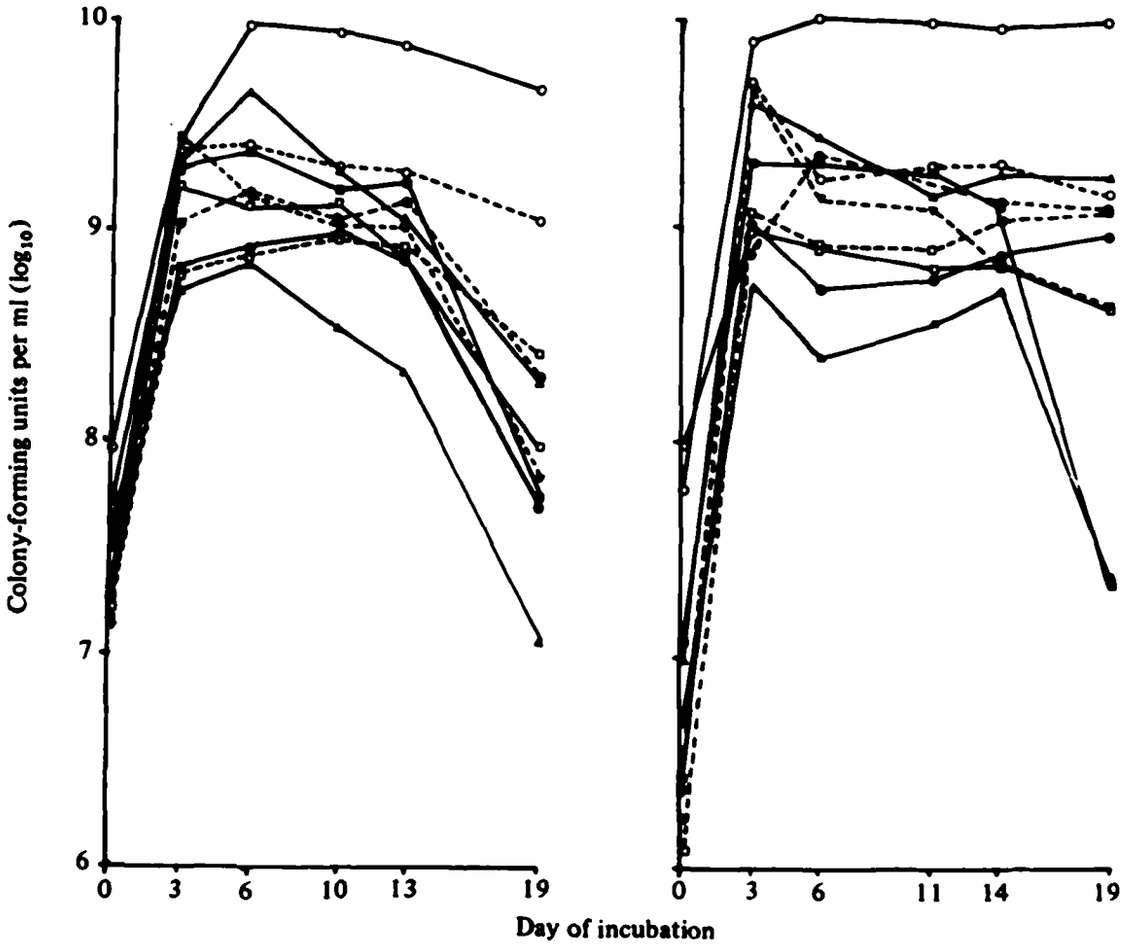

Fig. 1. Two similar experiments showing growth curves of LC strains: Y goat (○---○), Cov 2 (□——□), 74/2488 (□---□), 143-A66 Conn (■——■), Mankefår 2833 (○——○), 2605-Razi (▽---▽), Vom/Plum Island (▽——▽), Ghaleh Morghi-16 (●---●), F 30 (●——●); and *M. mycoides* subsp. *capri* Smith 1423 (▲——▲).

capri cultures ranged from 6·1 to 7·1 (mean 6·42); a colour change to full yellow occurred in three of the six cultures. After 14 days' incubation viable organisms were present in all except one (143–A66 Conn) of the 27 mycoplasma cultures.

Sensitivity to antibiotics

General reactions

The zones of inhibition, in mm, given by 16 mycoplasma strains (13 LC, 2 SC, and 1 subsp. *capri*) were as follows (range, with mean in brackets): cephaloridine 5 μg, 0; chloramphenicol 50 μg, 9–20 (13); colistin sulphate 10 μg, 0; fusidic acid 10 μg, 2–8 (5); lincomycin 2 μg, 1–9 (4); nalidixic acid 30 μg, 0; nitrofurantoin 200 μg, 8–22 (15); novobiocin 5 μg, 1–5 (3); streptomycin 25 μg, 0–7 (3); sulphafurazole 500 μg, 0; tetracycline, 10 μg, 9–17 (11). Four strains (Cov 2, Vom/Plum Island, 222–69 N.Y., Smith 1423) were apparently unaffected by the streptomycin disks.

Definitive test with streptomycin

Because streptomycin showed some promise of being of discriminative value, 27 mycoplasma strains (8 SC, 13 LC, and 6 subsp. *capri*) were tested for growth on blood agar plates containing doubling concentrations of the antibiotic.

The LC and subsp. *capri* strains all gave growth graded + + or + in the presence

of streptomycin 39·1 μg/ml, whereas all except one of the SC strains failed to give even reduced growth. In the presence of streptomycin 312·5 μg/ml six LC and four subsp. *capri* strains still gave growth (++ or +). Strains Smith 1423 and BQT (subsp. *capri*) and Vom/PI resisted streptomycin 10000 μg/ml. Of the eight SC strains, four gave '+' growth and all '++' growth in streptomycin 19·5 and 2·5 μg/ml respectively. Thus, in general, the LC and subsp. *capri* strains were more resistant than the SC strains. A confirmatory experiment, although giving results that varied in minor detail from those described above, bore out this conclusion.

DISCUSSION

Growth curves clearly distinguished strain Mankefår 2833 from the other LC strains examined. This was of particular interest in view of the distinctive characters of this strain, found by mycoplasmaemia and cross-protection tests and mentioned already. The exceptionally robust growth properties of Mankefår 2833 may account for its ability – in our experience unique amongst LC strains – to produce protracted mycoplasmaemia when doses of approximately 10^6 viable organisms are injected intraperitoneally into mice. If so, it would seem that Mankefår 2833 and the SC strains of *M. mycoides* subsp. *mycoides* – whose growth is much less robust – produce mycoplasmaemia by different mechanisms. The growth curves of the other LC strains examined did not differ strikingly from each other, except that strain 143-A66 Conn lost a great deal of its viability between the 13–14th and 19th days of incubation; this strain had also been shown in mouse experiments to possess unusual properties (Smith *et al.* 1980). The viable count of the subsp. *capri* strain was appreciably lower than that of the LC strains for much of the observation period.

Fermentation tests with 27 substrates gave results that were uniform in all but the following two respects. Sorbitol was fermented by all the LC and subsp. *capri* strains tested, though to varying degrees and not always sufficiently strongly to produce a complete colour change of bromothymol blue to full yellow; sorbitol was unaffected by the SC strains tested. The LC strains differed from the SC strains and subsp. *capri* in producing densely turbid growth – almost invariably dead by the 14th day of incubation – in the presence of readily fermented substrates.

All strains showed closely similar reactions in sensitivity tests with 11 antibiotics, except that the LC and subsp. *capri* strains were in general more resistant than SC strains to streptomycin.

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