Studies on an untyped avian mycoplasma related to *Mycoplasma gallisepticum*

A thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy.

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To my wife, *Esra* without whose loyal support, patience and love the work of this thesis would not have been possible.

To my children, *Basma* and *Sara* for their love.

PREFACE

The experimental work described in this thesis was undertaken in the Department of Veterinary Pathology, Jordan Building, University of Liverpool between 1987 and 1990.

ABSTRACT

This thesis describes studies on two related mycoplasma strains (4229 and B2/85) from a duck and partridge respectively. They were originally identified as *M.gallisepticum* but later serological studies on strain 4229 had revealed only a partial relationship with *M.gallisepticum*, and DNA:DNA hybridization showed approximately 40% homology between them, suggesting that the organism is a distinct and possibly new species. The purpose of this study was therefore to characterize strain 4229 according to the recommendations of the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* to establish it as a new species. Strain B2/85 was also included for comparison.

Restriction endonuclease analysis of the DNA of strains 4229 and B2/85 with three enzymes failed to show any differences between them, although they differed from *M.gallisepticum* strains S6 and PG31. Light and electron microscopy revealed a pleomorphic nature and absence of a cell wall. Both strains possessed a triple-layered membrane and a terminal tip-like organelle resembling that of *M.gallisepticum*. There was no evidence of helical forms. The organisms were not obligate anaerobes, they produced colonies typical of *Mollicutes* and L-phase bacteria, but there was no reversion to bacterial forms on passage in medium without bacterial inhibitors. Both strains passed through membrane filters of 450 and 220 nm pore diameter. They were therefore classified as members of class *Mollicutes*, but excluded from the order *Anaeroplasmatales* and from the family *Spiroplasmataceae*.

Sensitivity to digitonin and sodium polyanethol sulphonate gave indirect evidence of a sterol requirement, excluding the organisms from the order *Acholeplasmatales*. They were thus assigned to the order *Mycoplasmatales*, family *Mycoplasmataceae*. Since they did not hydrolyse urea they were placed in the genus *Mycoplasma*.

Biochemical tests were carried out to provide a species description. Both strains showed the same properties as *M.gallisepticum*. They were glucose positive, but negative for arginine hydrolysis, phosphatase activity, film and spots production and liquefaction of serum. Tetrazolium was reduced aerobically and anaerobically and there was haemadsorption and haemagglutination. Growth inhibition and indirect fluorescent antibody tests confirmed that strains 4229 and B2/85 were closely related to one another but serologically distinct from all the recognized avian and mammalian *Mycoplasma* species except *M.gallisepticum*.

Strains 4229 and B2/85 caused ciliostasis in chick and duck tracheal organ cultures, although they appeared less pathogenic than *M.gallisepticum*. Immunofluorescence demonstrated accumulation of the mycoplasmas on the epithelial mucosa, and transmission electron microscopy showed that both strains attached to the epithelial cell surface via their terminal tip structures in a similar way to *M.gallisepticum*. Both strains caused mortality of chick and duck embryos but were less pathogenic than *M.gallisepticum*.

One day old chickens and turkeys infected with strain B2/85 showed no signs of disease apart from slight transient respiratory signs in a few turkeys. The organism was reisolated from respiratory tissues in greater numbers from the turkeys than the chickens. Antibodies were not detected in sera of infected birds at 3 and 6 weeks post-inoculation but positive rapid serum agglutination reactions were obtained after booster intravenous inoculation. There were cross-reactions with *M.gallisepticum* antigens in both RSA and ELISA tests.

Preliminary attempts to isolate spontaneous, UV light or NTG-induced haemadsorption negative mutants of *M.gallisepticum* were not successful due to technical difficulties.

ACKNOWLEDGMENTS

I wish to express my sincere thanks and deep indebtedness to my supervisor Dr J.M.Bradbury for her guidance, constant encouragement, enthusiasm, and her support throughout the course of my project.

I would like also to thank Drs F.T.W.Jordan, R.C.Jones, and J.Varley and all other staff members of this laboratory for their assistance and constructive criticisms.

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My grateful thanks also for the encouragment and advice of Dr Malcolm Bennett and for the use of facilities in his laboratory. My sincere thanks also are due to Abdulrahman S.Al-Ankari for his assistance and co-operation.

I am extremely grateful for the valuable advice and help of Mr Gordon Ross with the electron microscopy studies and photographs.

Finally, I would like to thank all the friends who supported me and my family financially during writing up this thesis. I am particularly indebted to Mrs Susan Spencer, Mrs Eileen Ward, Mrs Debbie McInley and the members of Little Neston Methodist Church for their help and kindness.

ABBREVIATIONS

Blood agar	BA
Chicken infusion broth	CIB
Colony forming units	CFU
Growth inhibition	GI
Haemadsorption	HAd
Haemadsorption-negative	HAd^-
Haemagglutination	HA
Haemagglutination inhibition	HI
Indirect fluorescent antibody	IFA
International Committee on Systematic Bacteriology	ICSB
M.gallisepticum strain S6 of low passage	S6LP
M.gallisepticum strain S6 of high passage	S6HP
Mycoplasma agar	MA
Mycoplasma broth	MB
Organ culture medium	OCM
Phosphate buffered saline	PBS
Rapid serum agglutination	RSA
Sodium dodecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE
Tracheal organ culture	TOC
Transmission electron microscopy	TEM
Turkey infusion broth	TIB

Publications and presentations

The following papers represent parts of the work described in this thesis.

1. "An avian mycoplasma related to *Mycoplasma gallisepticum*: pathogenicity studies in tracheal organ cultures and embryonated eggs". Presented at the Annual Conference of the Association of Veterinary Teachers and Research Workers, Scarborough, 1990.

2. "Pathogenicity studies on an avian mycoplasma related to *M.gallisepticum*". Osama M.Saed, G.Ross and Janet M.Bradbury. Presented at the 8th Conference of the International Organization for Mycoplasmology, Istanbul, Turkey, 1990.

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Chapter 1

General introduction

The *Mollicutes* (trivial name 'mollicutes') form a distinct class of the smallest and simplest filterable prokaryotic organisms which are capable of self-replication in cell-free medium. They have several characters that differentiate them from other prokaryotes and from the viruses (Table 1:1).

The *Mollicutes* share general characteristics and cellular organisation with the bacteria, having 70S ribosomes and a typical prokaryotic circular chromosome which is folded, but a major distinction is the lack a rigid cell wall in mollicutes. This lack of a cell wall resulted in them being placed in a separate class, the *Mollicutes* (*mollis*, soft; *cutis*, skin), within the kingdom *Prokaryotae* (Edward and Freundt, 1967). The absence of a true cell wall and incapability of the organisms to synthesize peptidoglycan or its precursors was the recognized fundamental property of the organisms that were included in the class. Subsequently they were placed in a separate division, the *Tenericutes* (*tener*, soft; *cutis*, skin) (Gibbons and Murray, 1978).

Members of the division *Tenericutes,* class *Mollicutes* are thus defined by their lack of a rigid cell wall and inability to synthesize peptidoglycan precursors. As a consequence they are resistant to penicillin and its analogues. The cells are bounded only by a unit membrane, the plasma membrane, and are therefore highly pleomorphic. The organisms vary in shape from spherical or pear-shaped forms of 300 to 800 µm diameter to filamentous forms or helical filaments. Most mollicutes seem to be non-motile although some species show gliding motility, and those occurring as helical filaments show rotary, flexional and translational motility. The organisms are Gram negative.

Table 1:1 The relationships between the class *Mollicutes* and other microorganisms

Characters	Bacteria	Mollicutes	Chlamydia	Rickettsiae	Viruses
Cell-free medium	+	+	-	-	-
Generate metabolic energy	+	+	-	+	-
Independent protein synthesis	+	+	+	+	-
DNA & RNA	+	+	+	+	-
Reproduce by fission	+	+	+	+	-
Cell wall	+	-	+	+	-
Sterol requirement	-	+[1]	-	-	-

1. Except genus *Acholeplasma* and genus *Asteroleplasma*.

In vitro cultivation requires complex medium and most species form characteristic 'fried egg' colonies on agar.

Members of the class *Mollicutes* have a genome size of 5×10^8 to 1×10^9 daltons making them amongst the smallest recorded in the prokaryotes. The G + C content of the DNA is low and ranges from approximately 23 to 41 mol %.

As many of the bacteria are capable of producing wall-deficient growth forms (L-phase variants) which are similar in morphology to the naturally occurring mollicutes, two general hypotheses were earlier proposed to explain the relationship of mycoplasmas to other microorganisms (Edward, 1960; Klieneberger-Nobel, 1960; Dienes, 1963). In the first, the mycoplasmas were thought to represent a true biological class whose members were related to one another through evolution. In the second, it was suggested that mycoplasmas were a collection of wall-deficient forms derived from various bacteria. It was thought that, if the first hypothesis were correct, the mollicutes must represent the surviving descendants of exceedingly primitive bacteria i.e. those organisms which must have been present before the development of bacterial wall peptidoglycan synthesis. Moreover, such organisms might also have had smaller genomes than the contemporary bacteria (Neimark, 1979).

Phylogenetic studies of the *Mollicutes* based on nucleic acid investigations have been performed subsequently by a number of workers including Fox *et al.* (1980) and Woese *et al.* (1980). In particular, the comparative analysis of the base sequences of oligonucleotide catalogues of mollicute 16S ribosomal RNA (rRNA) has contributed to the understanding of the phylogenetic relatedness among higher taxa in the *Mollicutes*. These studies demonstrated that rRNAs were highly conserved during evolution and they showed the close relationship of *Mollicutes* to

the Gram-positive bacteria, the closest relatives being in the small subgroup of the clostridia represented by the two species, *Clostridium innocuum* and *Cl.ramosum*.

More recently Weisburg and co-workers (1989) have extended the analysis of 16S rRNA sequences to more than 40 species of the class *Mollicutes* and six of their walled relatives. Five groups of mollicute were recognised. These were named provisionally and are: the 'hominis group' which includes species such as *Mycoplasma hominis, M.lipophilum, M.pulmonis* and *M.neurolyticum*; the 'pneumoniae group' which includes species such as *M.pneumoniae*, *M.muris* and *M.gallisepticum*; the 'spiroplasma group' which includes *Spiroplasma citri, S.apis* but also *M.mycoides* subsp. *mycoides*; the 'anaeroplasma group', including anaeroplasmas and acholeplasmas; and the fifth group which contains only one species (*Asteroleplasma anaerobium*). The studies indicated the occurrence of independent genome reductions in the *Mollicutes* and that both *Mycoplasma* and *Acholeplasma* genera are polyphyletic. The authors considered that, although it has been known for many years that bacterial phenotypic properties are poor indicators of phylogenetic relationships, some phenotypic characters are useful to confirm the phylogenetic groups which have been established on the basis of genotypic characteristics such as rRNA sequences.

A report on reconciliation of approaches to bacterial systematics was made by an *Ad Hoc* Committee of the International Committee on Systematic Bacteriology in 1987. Conclusions and recommendations were based on reexamination of the bacterial nomenclature system in view of new taxonomic developments. It was concluded that an ideal taxonomy would involve one system, that the complete DNA sequence would be the reference standard for determining phylogeny, and that phylogeny should determine taxonomy. A further ideal was that nomenclature should agree with genomic information. The phylogenetic definition of a species would include strains with approximately 70% or greater DNA-DNA relatedness. There was an

overall concern that any phylogenetically based taxonomic scheme should show phenotypic consistency. However, the Subcommittee on the Taxonomy of *Mollicutes* (1991a) concurred that 'in spite of an important predictive value and the advantage of the phylogenetic schemes in organizing research perspectives, they lack the stability that is required in the taxonomic scheme'. The Subcommittee considered that efforts should be made to incorporate phylogenetic considerations into taxonomic classification but that their application could not be mandatory.

Until recently genome size was considered to be an important criterion in the higher classification of the *Mollicutes* due to bimodal clustering around 500 MDa and 1000 MDa. However, data obtained by pulsed-field gel electrophoresis (PFGE) (Pyle, *et al.*, 1988; Neimark and Lange, 1990) has revealed that a number of intermediate values exist and it has now been recommended that genome size be abandoned as a characteristic for determination of the higher taxa in the class *Mollicutes* (Subcommittee on the Taxonomy of *Mollicutes* , 1991b).

It has been shown that a common phenotypic character of the *Mollicutes* is rifampicin insensitivity and furthermore this was demonstrated in the phylogenetically related *Clostridia* species (Gadeau *et al.*, 1986; Pellegrin *et al.*, 1990). The Subcommittee on the Taxonomy of *Mollicutes* has suggested that revision of their document 'Proposal of minimal standards for the description of new species of the class Mollicutes' (Subcommittee on the Taxonomy of *Mollicutes*, 1979) may eventually include rifampicin insensitivity in the definition of the class (Subcommittee on the Taxonomy of *Mollicutes* , 1991a).

At present, three orders are recognized within the the class *Mollicutes* as shown in Table 1:2. These are the *Mycoplasmatales*, the *Acholeplasmatales* and the *Anaeroplasmatales*. An earlier proposal to elevate the family *Spiroplasmataceae* to ordinal rank (Subcommittee on the Taxonomy of *Mollicutes*, 1988) has been held in

Table 1:2 Taxonomy of the class *Mollicutes*

Order I	***Mycoplasmatales***
Family I	*Mycoplasmataceae*
Genus I	*Mycoplasma* (sterol requiring)
Genus II	*Ureaplasma* (sterol requiring, catabolise urea)
Family II	*Spiroplasmataceae*
Genus I	*Spiroplasma* (sterol requiring, helical morphology)
Order II	***Acholeplasmatales***
Family I	*Acholeplasmataceae*
Genus I	*Acholeplasma* (non-sterol requiring)
Order III	***Anaeroplasmatales*** (strictly anaerobic)
Family I	*Anaeroplasmataceae*
Genus I	*Anaeroplasma* (sterol requiring)
Genus II	*Asteroleplasma* (non-sterol requiring)

abeyance pending further clarification of mollicute phylogeny (Subcommittee on the Taxonomy of *Mollicutes*, 1991a).

The order *Mycoplasmatales* includes two families, the *Mycoplasmataceae* and the *Spiroplasmataceae* , members of which exhibit dependence on sterol for growth. The family *Mycoplasmataceae* contains two genera, *Mycoplasma* and *Ureaplasma* which are differentiated by the ability of members of the genus *Ureaplasma* to hydrolyse urea. Approximately 90 *Mycoplasma* species are now recognised and have been found in mammals, birds, fish, insects and plants. There are five named species of *Ureaplasma* , all isolated from mammals or birds.

The family *Spiroplasmataceae* contains the genus *Spiroplasma* and is distinguished by the combination of sterol requirement, helical morphology and motility (Subcommittee on the Taxonomy of *Mollicutes*, 1979). So far 11 species are recognised in the genus and all have been isolated from either plants or arthropods.

Species belonging to the family *Acholeplasmataceae* , genus *Acholeplasma* differ from species of the other families of the class *Mollicutes* in many major respects, including lack of a nutritional requirement for sterol, the ability of most species to synthesize saturated fatty acids and polyterpenes from acetate, and several other factors related to lipid metabolism and to the incorporation and location of lipids in the cell membranes. Thus a new order, *Acholeplasmatales* was proposed (Freundt *et al.*, 1984). Members of the genus *Acholeplasma* seem to have less host specificity than other genera. There are 12 recognised species and the organisms have been isolated from mammals, birds, insects and plants.

Members of the order *Anaeroplasmatales* , family *Anaeroplasmataceae* are strictly anaerobic. They have been isolated from the rumen of cattle and sheep. The family contains two genera the *Anaeroplasma* and the *Asteroleplasma*. Members of the

genus *Anaeroplasma* require sterol for growth while members of *Asteroleplasma* do not (Robinson and Freundt, 1987). So far there are four recognised species of *Anaeroplasma* and the one species of *Asteroleplasma*.

Species description in the *Mollicutes* depends upon biochemical and serological characteristics. The genus *Mycoplasma*, with approximately 90 members (trivial name 'mycoplasmas'), contains more species than any other genus in the class. In spite of the large numbers of species found in animals and humans, relatively few of them are known to be pathogens.

The first reports of isolation of avian mycoplasmas were probably from chickens by Nelson (1936; 1939) who demonstrated coccobacilliform bodies in nasal exudates from birds with coryza. Markham and Wong (1952) cultivated 'pleuropneumonia-like organisms' from chickens and turkeys and considered them to be the causative agent of 'chronic respiratory disease' in chickens and of 'infectious sinusitis' in turkeys. These organisms and other isolates made at the time were thought to be serologically similar, but later Adler *et al.* (1957) distinguished two serotypes. Yamamoto and Adler (1958) characterized five distinct serotypes which they designated I to V and Kleckner (1960) added another three, now designating them A to H. Yoder and Hofstad (1964) identified four more serotypes thus increasing the total number to twelve and the designation to L. Dierks *et al.* (1967) reported 19 serotypes designated from A to S. However, further biochemical and serological studies resulted in redefinition and a reduction of the number of serotypes to ten (Barber and Fabricant, 1971; Aycardi *et al.*, 1971; Frey *et al.*, 1972). To these were added two new *Mycoplasma* species from pigeons (Shimizu *et al.*, 1978), and Jordan *et al.* (1982) later provided species descriptions and names for several of the earlier unnamed serotypes. Bradbury and co-workers then described a further four new *Mycoplasma* species (Bradbury *et al.*, 1983; Forrest and Bradbury, 1984;

Bradbury and Forrest, 1984; Bradbury *et al.*, 1988a) bringing the total number of recognised *Mycoplasma* species to 17 (Table 1:3).

Members of the genera *Acholeplasma* and *Ureaplasma* also occur in birds. For example *Acholeplasma laidlawii* has been isolated from chickens, turkeys, geese, pigeons and ducks (Stipkovits *et al.*, 1975; Amin, 1977; Jordan and Amin, 1980; Bencina *et al.*, 1987; Tiong, 1990; Sabry *et al.*, 1990). *A.axanthum* has been isolated from goose embryos and laying geese (Stipkovits *et al.*, 1975) and also from ducks (Fawzia, 1976; Bencina *et al.*, 1987; Sabry *et al.*, 1990). Other acholeplasmas (*A.oculi* and *A.modicum*) have also been recently isolated from ducks (J.M.Bradbury, personal communication).

Ureaplasmas have been isolated from chickens and jungle-fowl (Stipkovits and Rashwan, 1976; Koshimizu and Magaribuchi, 1977) and from turkey semen (Stipkovits et al., 1978). The Japanese isolates were later classified as *Ureaplasma gallorale* (Koshimzu et al., 1987).

Among the *Mollicutes* found in avians, only four have been clearly established as pathogens of economic importance for domestic poultry due to their association with disease and decreased production (Jordan, 1990). All are members of the genus *Mycoplasma*. They are *M.gallisepticum* and *M.synoviae* in the chicken and the turkey, and *M.meleagridis* and *M.iowae* in the turkey.

On a world-wide basis *M.gallisepticum* is the most important of the pathogenic avian *Mycoplasma* species. Infections with this organism occur naturally in chickens and turkeys (Yoder, 1991) but isolations associated with disease have been also reported from other avian hosts such as pigeon (Bencina *et al.*, 1987), pheasant (Van Roekel and Olesiuk, 1953; Osborn and Pomeroy, 1958; Reece *et al.*, 1986), partridge (Wichmann, 1957; Yoder and Hofstad, 1964; Reece *et al.*, 1986), peacock (Wills,

Table 1:3 The avian *Mycoplasma* species

Species	Type strain	Common hosts
M.anatis	1340	duck
M.anseris	1219	goose
M.cloacale	383	turkey, goose
M.columbinasale	694	pigeon
M.columbinum	MMP1	pigeon
M.columborale	MMP4	pigeon
M.gallinaceum	DD	chicken
M.gallinarum	PG16	chicken, turkey
M.gallisepticum	PG31	chicken, turkey
M.gallopavonis	WR1	turkey
M.glycophilum	486	chicken
M.iners	PG30	chicken, turkey
M.iowae [1]	695	turkey, chicken
M.lipofaciens	R171	chicken, turkey
M.meleagridis	17529	turkey
M.pullorum	CKK	chicken
M.synoviae	WVU1853	chicken, turkey

1. This species also includes serovars J, K, N, Q and R

1955; Kleven *et al.*, 1988b), quail (Madden *et al.*, 1967; Tiong, 1978; Reece *et al.*, 1986; Bencina *et al.*, 1987), guinea fowl (Van Roekel and Olesiuk, 1953) and parrot (Bozeman *et al.*, 1984).

M.gallisepticum has also been reported to occur in geese (Bencina *et al.*, 1988a) and in ducks (Jordan and Amin, 1980; Bencina *et al.*, 1988b) but the pathogenicity has not been clearly established.

In 1984, an avian mycoplasma, designated strain 4229, was isolated from the turbinate of a 'mule' (broiler) duck with airsacculitis and pericarditis by Vuillaume and co-workers in south-west France. It was identified as *M.gallisepticum* by growth inhibition tests in France (Dupiellet, 1984) and by immunofluorescence in our laboratory (J.M. Bradbury, personal communication).

Four similar isolates were recovered from adult geese (Buntz *et al.*, 1986; Buntz, 1987) and again all four isolates were identified by growth inhibition in France and by immunofluorescence in our laboratory as *M.gallisepticum*. In 1985 a similar mycoplasma was isolated in England by J.M.Bradbury from the eye of a partridge with typical *M.gallisepticum* 'infectious sinusitis'. This strain (designated B2/85) was also identified by immunofluorescence as *M.gallisepticum*.

Dupiellet and co-workers (Dupiellet, 1988; Dupiellet *et al.*, 1990) compared the duck isolate and three goose isolates with well characterized strains of *M.gallisepticum* (the type strain PG31 and strain S6) using serological and molecular techniques. Although a serological cross-reaction between the duck and goose strains and the *M.gallisepticum* strains was observed by growth and metabolism inhibition tests, the reactions were weaker than might be expected within the same species. Restriction enzyme analysis of the DNA, and SDS-polyacrylamide gel electrophoresis analysis (SDS-PAGE) of the proteins of the organisms showed homogeneity between the

duck and goose strains but obvious differences between them and the *M.gallisepticum* strains. Furthermore the G + C contents of the duck and goose strains were consistently approximately 2 mol% lower than those of the *M.gallisepticum* strains. These findings gave rise to doubts about the true relationship of the duck and goose strains to *M.gallisepticum*, and subsequent DNA:DNA hybridization studies demonstrated a high level of homology (96%) between duck and goose strains but a much lower relationship (38-46%) between them and *M.gallisepticum*. As a result of these studies the authors suggested that the duck and goose strains should be considered as separate species.

The above serological and molecular findings were supported by the observations of other workers (Yogev *et al.*, 1988a and b) who used genomic fingerprinting with rRNA and *tuf* gene probes to determine the intraspecies heterogeneity between *M.gallisepticum* strains. Strains 4229, and B2/85 (and a goose strain 30902) were included in these studies because at that time they were still thought to be *M.gallisepticum*. It was found that these 'variant' strains shared a unique fingerprint which was clearly different from those of a number of well-known strains of *M.gallisepticum*.

Dupiellet (1988) used two rRNA probes to analyse strain 4229 and two goose strains and to compare them with *M.gallisepticum*. Like Yogev and co-workers, he found that the duck and goose isolates gave identical patterns which were distinguishable from those of *M.gallisepticum*.

The epidemiological and pathological impact of strain 4229 and the goose and partridge strains in poultry is not known. Preliminary *in vivo* and *in vitro* investigations by Buntz (1987), Dupiellet (1988) and Dupiellet *et al.*, (1990) suggested that the goose and duck strains may be pathogenic for these hosts and their embryos, and there was some evidence of pathogenicity of strain 4229 for live

turkeys and for chicken embryos. The pathogenicity of strain B2/85 was not investigated.

Apart from their potential pathogenicity, these strains may present an important diagnostic problem due to their serological relatedness to *M.gallisepticum*. This is clearly demonstrated by the fact that the isolates were originally identified as *M.gallisepticum* by us and by colleagues in France.

The present studies were designed to characterize this organism, represented by strain 4229, according to the criteria recommended by the Subcommittee on the Taxonomy of *Mollicutes* (1979), to determine its proper position within the class, and to determine the properties for its description as a proposed new avian species of the class *Mollicutes*. It was decided to include strain B2/85 for comparative purposes since it had been isolated from a different host and in a different country to 4229 but appeared to be a similar organism. Thus an initial step was to compare these two strains by restriction enzyme analysis of their DNA. Following this, full taxonomic characterization of the potential new species would 'be undertaken, and then further clarification of its pathogenicity carried out using tracheal organ cultures, embryonated eggs and chickens and turkeys.

During the course of these studies it was found that strains 4229 and B2/85 possessed attachment organelles similar to those seen in *M.gallisepticum*. In view of the possible importance of such structures as mediators of the first step of infection, it was decided to initiate investigations into their role by attempting to derive haemadsorption negative mutants, following the same approaches already used for *M.pneumoniae*. *M.gallisepticum* was chosen for these pilot studies because of its recognition as an important pathogen. These investigations are presented in a short final Chapter.

Chapter 2 General Materials and Methods

Chapter 2 General Materials and Methods

The materials and methods that were used throughout these experimental studies are described in this section, whereas others are described in the appropriate section.

2.1 Glassware

All used glassware except pipettes was collected in stainless steel buckets and autoclaved at a pressure of 15 lb/sq.in (121°C) for 15 minutes. Bottles contaminated with blood were soaked overnight in 2% of liquid detergent (Micro[1]). The glassware was either scrubbed using a brushing machine[2] and then rinsed manually in successive changes of tap, distilled and deionized water, or it was washed in a glass washing machine[3] with Hamo detergent[4] followed by serial rinsing in tap, distilled and deionized water. The glassware was dried in a hot air oven and then bottles were capped with plastic or metal caps, and flasks, cylinders and test tubes with aluminium foil. The glassware was sterilized either by autoclaving for 15 minutes at 15 lb/sq.in or by heating in a hot air oven at 160°C for at least 2 hours.

Used graduated and Pasteur pipettes were disinfected overnight in a 1% (v/v) solution of sodium hypochlorite (Chloros[4]). Pasteur pipettes were then discarded but graduated pipettes were recycled. The cotton wool plugs were removed and the pipettes were soaked overnight in a 2% (v/v) solution of Micro. They were rinsed in an automatic pipette-washer using running tap water for at least 2 hours and then

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1 International Products Corporation, Kent, U.K.

2 The Thomas Hill Engineering Co Ltd., U.K.

3 Jean Gallay and Co Ltd., Hemel Hempstead, Herts, U.K.

4 Golden Grain Products Ltd., Liverpool, U.K.

placed upright in a container of distilled water for at least 1 hour. They were dried, plugged with cotton wool and sterilized in metal containers in a hot air oven.

2.2 Preparation and storage of media ingredients

Swine serum

Swine serum was obtained by collection of blood from a local abbatoir. It was left overnight at room temperature and then the serum was removed and centrifuged at 1,000 g for 20 minutes to sediment the red blood cells. The supernatent serum was removed and passed through a clarifying filter (grade XE 150[1]) followed by a Seitz filter (grade XE 675[1]) for sterilisation. The serum was inactivated by heating at 56°C for 30 minutes and stored at -20°C.

Horse serum

Sterile horse serum[2] from a commercial source was stored at -20°C. It had been examined for mycoplasma contamination by the company before release.

Yeast Extract

A 25% (w/v) suspension of active dried bakers' yeast granules[3] was prepared in warm distilled water and heated to boiling point with continuous stirring. After cooling the yeast suspension was centrifuged at 2,200 g for 30 minutes and the supernatant was clarified by filtration (filter grade XE 150). The pH was adjusted to 8.0 with 1M NaOH before passage through a Seitz filter (grade XE 675). The yeast extract was stored at -20°C.

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1 Butts Mill, Bamoldwick, Lancashire, U.K.

2 Flow laboratories, Scotland

3 DCL company Ltd, Crown house, Surrey, U.K.

Commercial Yeast Extract

A 7% (w/v) solution of dried yeast granules (Oxoid L21)[1] was prepared in deionized water. It was dispensed in 100 ml amounts, autoclaved at 15 lb/sq.in for 15 minutes and stored at 4°C.

Frile Yeast Extract

A 13.3% (w/v) suspension of pure dried yeast (*Saccharomyces cerevisiae*, type II)[2] in distilled water was heated at 37°C for 20 minutes and then to 90-100°C for 5 minutes. After cooling, it was centrifuged at 1,000 g for 30 minutes and the supernatant was autoclaved at 10 lb/sq.in for 5 minutes and stored at -20°C.

SP-4 yeast extract

A 25% (w/v) suspension of pure dried yeast (*Saccharomyces cerevisiae*, type II) was added to deionized water, the mixture was steamed in a boiling water bath for 30 minutes and, after cooling, it was centrifuged at 2,200 g for 30 minutes. The supernatant was autoclaved at 15lb/sq.in for 15 minutes and stored at 4°C.

D-Glucose

Two solutions of glucose[3] in distilled water were used, one of 20% (w/v) and the other 10% (w/v). The pH was adjusted to 7.8-8.0 and the solutions were sterilized by Seitz filtration and stored at -20°C.

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1 Oxoid Ltd, Basingstoke, Hampshire, U.K.

2 Sigma Chemical company, St.Louis, MO, U.S.A.

3 BDH Chemicals Ltd., Poole, U.K.

L-Arginine monohydrochloride

A 10% (w/v) solution of L-arginine monohydrochloride[1] was prepared as described for glucose except that the pH was adjusted to 7.0. It was stored at -20°C.

Nicotinamide adenine dinucleotide (NAD)

A 1% (w/v) solution of nicotinamide adenine dinucleotide[2] was prepared in distilled water, sterilized by Seitz filtration (grade XE 675) and stored at -20°C.

Thallium acetate

A solution of 5% (w/v) thallium acetate[1] was prepared in distilled water, sterilized by Seitz filtration (grade XE 675) and stored at -20°C.

Phenol red

Phenol red powder[1] (0.1 g) was combined with 2.82 ml of 0.1 M NaOH and thoroughly mixed using a pestle and mortar. The mixture was then made up to 100 ml with distilled water, sterilized by autoclaving at 10 lb/sq.in for 30 minutes, and stored at 4°C.

Penicillin

Crystalline penicillin (sodium salt)[3] solution was prepared in sterile distilled water to contain 200,000 international units (iu) /ml. The solution was stored at 4°C for up to seven days.

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1 BDH Chemicals Ltd., Poole, U.K.

2 Boehringer Mannheim GmbH, Germany

3 Glaxo laboratories Ltd., Greenford, U.K.

Ampicillin

A 40 µg/ml solution of Penbritin[1] was prepared in sterile distilled water and stored at -20°C.

2.3 Preparation and storage of culture media

Mycoplasma broth (MB)

The medium described by Bradbury (1977) based on that of Taylor-Robinson *et al.* (1966) was used in the form of broth and agar for culturing most of the mycoplasmas in this study.

Part A	
Bacto-PPLO broth powder (w/o crystal violet)[2]	1.47 g
Distilled water	70.0 ml
Part B	
Swine serum (inactivated)	15.0 ml
Yeast extract (25% w/v)	10.0 ml
L-arginine (10% w/v)	1.0 ml
NAD (1% w/v)	1 0 ml
D-glucose (10% w/v)	1.0 ml
Penicillin (200,000 iu/ml)	0.5 ml
Phenol red (0.1% w/v)	2.0 ml

Part A was autoclaved at 15 lb/sq.in for 15 minutes and cooled to below 56°C. The ingredients of part B were mixed aseptically, with the solution of thallium acetate

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[1] Beecham Animal Health, Brentford, Middx, U.K.

[2] Difco Laboratories, Detroit, Michigan, U.S.A.

added before the swine serum to avoid precipitation. Parts A and B were then combined and stored at 4°C for up to one month.

Mycoplasma agar (MA)

This was prepared as for MB except that it included 1.0 g Lab M agar No.2 powder[1] in part A and, after autoclaving, the molten agar was cooled to 56°C and maintained at that temperature in a water bath. The ingredients of part B, omitting phenol red, were mixed and heated to 56°C. Parts A and B were mixed and dispensed 8 ml quantities in 5 cm diameter sterile plastic Petri dishes[2]. The agar was allowed to solidify and excess moisture was removed by drying for 10-12 hours at room temperature. Plates were then stored at 4°C for up to one month.

Horse serum broth

This broth and corresponding agar were recommended by Dr R.H. Leach (Colindale, London) for growth of some mammalian mycoplasmas. In our study it proved useful for the cultivation of *M.sualvi* and *M.faucium* (under anaerobic conditions), and also *M.pneumoniae* and *M.mobile*.

Part A

Mycoplasma Broth Base (CM403)[3] (25.5 g) was dissolved in 1 litre of deionized water, dispensed in 70 ml amounts and autoclaved at 15 lbs/sq.in for 15 minutes. It was stored at room temperature.

Part B

Horse serum (unheated)	20.0 ml
Yeast extract (Commercial)	10.0 ml
Thallium acetate (1% w/v)	2.5 ml

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1 Lab M, Topley house, Bury, Lancs, U.K.

2 Sterilin Ltd, Hounslow, U.K.

3 Oxoid Ltd, Basingstoke, U.K.

Penicillin (100,000 iu/ml)	0.2 ml
Phenol red (0.2%)	1.0 ml

The sterile ingredients of part B were mixed and 70 ml of part A added. The broth was stored at 4°C.

Horse serum agar

Part A

Mycoplasma Agar Base powder (CM401)[1] (35.5 g) was dissolved in 1 litre of deionized water, dispensed in 70 ml amounts, autoclaved at 15 lb/sq.in for 15 minutes and stored at room temperature.

Part B

Horse serum	20.0 ml
Yeast extract (commercial)	10.0 ml
Thallium acetate (1% w/v)	2.5 ml
Penicillin (100,000 iu/ml)	0.2 ml

The ingredients of part B were mixed aseptically, warmed to 50°C, and then mixed with 70 ml of part A which had previously been melted and allowed to cool to 50°C. The medium was dispensed into sterile 5 cm plastic Petri dishes in 8 ml volumes.

FF broth

This medium and the corresponding agar were described by Friis (1971) for cultivation of *M.hyopneumoniae* , *M.flocculare* and *M.dispar.* In our study they were successfully used to cultivate *M.dispar* and *M.neurolyticum* although growth of *M.dispar* was not consistent.

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[1] Oxoid Ltd, Basingstoke, U.K.

Part A	
Hanks balanced salt solution (10X)[1]	3.25 ml
Deionized water	76.0 ml
BBL-Brain Heart infusion[2]	0.82 g
Bacto-PPLO broth[3]	0.84 g
Part B	
Swine serum[1] (inactivated)	10.0 ml
Horse serum[4] (uninactivated)	10.0 ml
Friis yeast extract	5.0 ml
Thallium acetate (1% w/v)	1.0 ml
Phenol red (0.1% w/v)	1.0 ml
Ampicillin (40 ug/ml)	0.4 ml

The ingredients of part A were dissolved by boiling and then sterilized by autoclaving at 15 lb/sq.in for 5 minutes. Part B was assembled and the pH adjusted to 7.8 before adding to cooled part A

FF agar

This medium was prepared by addition 0.7 g of agarose '10' (Electran)[5] to part A as described in the broth medium and, after cooling to 56°C, the mixture was added to part B which had been preheated to 56°C.

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1 Gibco, Life Technologies Ltd., U.K

2 BBL, Microbiology Systems, Becton Dickinson & Co., Cockeysville, MD, U.S.A.

3 Difco Laboratories, Detroit, Michigan, U.S.A.

4 Flow Laboratories, U.K

5 BDH Chemicals Ltd., Poole, U.K.

SP-4 broth

SP-4 medium was first described by Tully *et al.* (1977) for isolation of *Spiroplasma* species and has since been found useful to cultivate a number of mycoplasma species including *M.pneumoniae, M.dispar, M.synoviae, M.fastidiosum, M.feliminutum, M.alvi,* and *M.sualvi* (Freundt, 1983a). It has also been used to cultivate *M.genitalium* (J.G.Tully, personal communication). In our studies it was used to grow *M.muris.*

Part A	
BBL-mycoplasma broth base[1]	0.35 g
Bacto-Tryptone[2]	1.0 g
Glucose	0.5 g
Bacto-Peptone[2]	0.53 g
Deionized water	61.0 ml
Part B:	
SP-4 yeast extract	3.5 ml
Yeastolate[2] (2%)	10.0 ml
Foetal bovine serum[3] (Inactivated)	17.0 ml
Penicillin (100,000 iu/ml)	0.5 ml
Phenol red (0.1% w/v)	1.0 ml
CMRL-1066 (10X)[4]	5.0 ml
Sodium bicarbonate (7.5%)[4]	5.0 ml
Glutamine (100X)[4]	0.2 ml

[1] BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD, U.S.A.
[2] Difco Laboratories, Detroit, Michigan, U.S.A.
[3] Flow Laboratories, U.K.
[4] Gibco, Life Technologies Ltd., U.K.

The ingredients of part A were dissolved, the pH was adjusted to 7.8. and the solution autoclaved at 15 lb/sq.in for 15 minutes. The ingredients of part B were sterilized by Seitz filtration and added to part A after it had cooled. The broth was stored at 4°C.

SP-4 agar

This was prepared by addition of 1.0 g of Noble agar[1] to part A above and, after cooling to 56°C in a water bath, it was mixed with part B preheated to the same temperature.

Serum fraction broth

Part A	
Bacto-PPLO broth powder	1.76 g
Distilled water	84.0 ml
Part B	
Bacto-PPLO serum fraction[1]	1.0 ml
D-glucose (10% w/v)	1.0 ml
L-arginine (10% w/v)	1.0 ml
Thallium acetate (5% w/v)	1.0 ml
Penicillin (200,000 iu/ml)	0.5 ml
Phenol red (0.1%)	2.0 ml

Part A was autoclaved at 15 lb/sq.in for 15 minutes and then cooled to 56°C. The sterile ingredients of part B were heated to 56°C and added to part A. The medium was stored at 4°C.

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[1] Difco Laboratories, Detroit, Michigan, U.S.A.

Blood agar

Blood agar base[1]	4.0 g
Distilled water	100.0 ml
Sheep blood (defibrinated)	7.0 ml

The blood agar base was dissolving in distilled water in a boiling water bath and was autoclaved at 15 lb/sq.in for 15 minutes. The agar was cooled to 56°C, and 7% of (v/v) sterile defibrinated sheep blood was added. The medium was dispensed in 5 cm diameter sterile plastic Petri dishes in 7 ml volumes and stored at 4°C for up to one week.

Sabouraud dextrose agar

Sabouraud agar[1]	6.5 g
Distilled water	100.0 ml

The agar powder was soaked in the distilled water for 15 minutes and then autoclaved at 15 lb/sq.in for 15 minutes. It was dispensed into 5 cm diameter sterile plastic Petri dishes and stored at 4°C for up to 1 week.

2.4 Diluents and reagents

Phosphate buffered saline (PBS)

To prepare 1 litre:

Sodium dihydrogen orthophosphate[2] ($NaH_2PO_4.2H_2O$)	0.39 g
Di-sodium hydrogen orthophosphate[2] ($Na_2HPO_4.12H_2O$)	1.07 g

[1] Oxoid Ltd., Basingstoke, U.K.
[2] BDH Chemicals Ltd., Poole, U.K.

Sodium chloride	8.5 g
Distilled water	1,000.0ml

The ingredients were dissolved in the distilled water and the pH was adjusted to 7.0 with 1M NaOH or 1M HCl. Sterilization, when required, was carried out by autoclaving at 15 lb/sq.in for 15 minutes.

Physiological saline

To prepare 100 ml:

Sodium chloride	0.85 g
Distilled water	100.0 ml

The solution was sterilized by autoclaving at 15 lb/sq.in for 15 minutes.

Citrate saline

To prepare 100 ml:

Sodium citrate	4.0 g
Sodium chloride	0.85 g
Distilled water	100.0 ml

The solution was autoclaved at 15 lb/sq.in for 15 minutes.

Carbonate-bicarbonate buffer

Sodium bicarbonate	21.0 g
Sodium carbonate	26.5 g
Distilled water	1,000.0ml

The pH was adjusted to 9.0.

2.5 Culture and storage of mycoplasmas

Routine culturing

The mycoplasma type strains and field strains were propagated in mycoplasma agar (MA) or broth (MB) with the following exceptions: *M.muris* was grown in SP-4 medium, *M.neurolyticum* and *M.dispar* were cultured in FF medium and *M.sualvi, M.faucium, M.mobile and M.pneumoniae* were grown in horse serum medium. Broths in screw-capped bottles were sealed with Parafilm[1] and incubated at 37°C except for *M.faucium* and *M.sualvi* which were incubated at 37°C in loosely sealed bottles in a Gas pak[2] anaerobic system with added nitrogen. Agar cultures of these two species were also incubated anaerobically while all other inoculated agar plates were incubated aerobically at 37°C in a candle jar.

Samples from experimental birds and organ culture fluids were also cultured using MB and MA plates incubated in candle jars. Plates were kept for 21 days before being discarded as negative. Subculture from broth onto agar was carried out as soon as any colour change in the pH indicator was seen.

Broth to broth subculture was carried out using a 10% (v/v) inoculum. A large airspace (50%) was allowed in the culture vessel except for the broths that were incubated anaerobically. Subculture from broth to agar was made by spreading broth onto the agar surface with a wire loop or a sterile pipette. The broth was allowed to absorb before the plates were inverted for incubation. Subculture from agar to agar was carried out by removing a colony-bearing agar block, inverting it on a fresh agar plate and pushing it across the surface. To subculture from agar to broth, a colony-bearing block was placed in 1 ml of broth.

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[1] American Can Co., Greenwich, CT, U.S.A.

[2] Oxoid Ltd., Basingstoke, Hampshire, U.K.

Storage

Cultures of both reference and field strains were stored at -60°C or -70°C either in 1 ml broth in screw cap glass vials or as colony-bearing agar blocks in 1 ml broth.

2.6 Cloning

The Subcommittee on the Taxonomy of *Mollicutes* (1979) recommends that preliminary cloning procedures are undertaken before carrying out characterization tests on a potential new species. Thus, the two strains, 4229 and B2/85, used in this study were cloned three times before any laboratory tests were undertaken. The cultures used for the embryo and live bird pathogenicity experiments were cloned once in order to keep the passage level to a minimum.

The cloning procedure was as described by Tully (1983a). A broth culture was passed by gentle filtration (usually in 1-2 ml amounts) through a membrane filter[1] with a pore diameter of 450 nm. Tenfold serial dilutions of the filtrate were made and 0.2 ml of each was inoculated onto MA. After incubation for 3-7 days at 37°C, a plate with well-dispersed colonies was selected, and using a sterile scalpel, a single colony was cut and transferred into 5 ml mycoplasma broth. It was incubated until growth was obvious by change in colour of the pH indicator and/or turbidity. This completed one cloning. The filtration procedure was repeated twice to provide three-times cloned cultures.

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[1] Sartorius, GmbH, D-3400 Gottingen, Germany

2.7 Mycoplasma strains and antisera

Strains 4229 and B2/85

Strain 4229 was isolated from the turbinate of a 'mule' duck in Southwest France and strain B2/85 from the eye of a partridge in the south of England. After their original isolation they were cloned by filtration (2.6) and their purity was confirmed by indirect immunofluorescence (see 6.2.2). Stocks were frozen in 1 ml aliquots after the first and third cloning. The *in vitro* passage level of strain 4229 was not known but it had undergone 6 passages here after the first cloning and 10 after the third. The total *in vitro* passage levels for strain B2/85 were 6 and 10 after one and three clonings respectively.

M.gallisepticum S6

A low and high passage culture of *M.gallisepticum* S6 were used in these studies and were designated S6LP and S6HP respectively. The strain was originally isolated from turkey brain (Zander, 1961) and was obtained from Dr D.H.Roberts, Central Veterinary Laboratory, Weybridge. It had subsequently been cloned three times and passaged in SPF turkeys in this laboratory (Power and Jordan, 1976). After reisolation the identity and purity was confirmed by immunofluorescence. Stocks of S6LP were grown up in sufficient volume for freezing for use in the experiments. Aliquots of 1 ml of the fourth *in vitro* passage were stored at -60°C.

The high passage strain (S6HP) was the same organism but had undergone 105 *in vitro* passages in MB and was then frozen in aliquots as above.

Type strains

The strains used are shown in Table 2:1. They were obtained from the former FAO/WHO Collaborating Centre for Animal Mycoplasmas, University of Aarhus, Denmark, with the exception of *M.ellychniae*, *M.fastidiosum*, *M.genitalium*,

Table 2:1 The reference mycoplasma species and type strains

M.alkalescens D12
M.alvi Ilsley
M.anatis 1340
M.anseris 1219
M.arginini G230
M.arthritidis PG6
M.bovigenitalium PG11
M.bovirhinis PG43
M.bovis Donetta
M.bovoculi M165/69
M.buccale CH20247
M.californicum ST-6
M.canadense 275C
M.canis PG14
M.capricolum California Kid
M.caviae G122
M.cavipharyngis 117C
M.citelli RG-2C
M.cloacale 383
M.collis 58B
M.columbinasale 694
M.columbinum MMP1
M.columborale MMP4
M.conjunctivae HRC581
M.cricetuli CH
M.cynos H831
M.dispar 462/2
M.edwardii PG24
M.ellychniae ELCN-1
M.equigenitalium T37
M.equirhinis M432/72
M.fastidiosum 4822
M.faucium DC333
M.felifaucium PU
M.feliminutum Ben
M.felis CO
M.fermentans PG18
M.flocculare Ms42
M.gallinaceum DD
M.gallinarum PG16
M.gallisepticum PG31
M.gallopavonis WR1
M.gateae CS
M.genitalium G37
M.glycophilum 486
M.hominis PG21
M.hyopneumoniae J
M.hyorhinis BTS-7
M.hyopharyngis H3-6B F
M.hyosynoviae S16
M.iners PG30
M.iowae 695
M.lipofaciens R171
M.lipophilum MaBy
M.maculosum PG15
M.meleagridis 17529
M.moatsii MK405
M.mobile 163K
M.molare H542
M.muris RIII4
M.mustelae MX9
M.mycoides subsp. *capri* PG3
M.mycoides subsp.mycoides PG1
M.neurolyticum A
M.opalescens MH5408
M.orale CH19299
M.ovipneunmoniae Y-98
M.pirum 70-159
M.pneumoniae FH
M.primatum HRC292
M.pullorum CKK
M.pulmonis PG34
M.putrefaciens KS-1
M.salivarium PG20
M.spumans PG13
M.sualvi Mayfield B
M.subdolum TB
M.synoviae WVU1853
M.testudinis 01008
M.verecundum 107
avian serovar J strain DJA
avian serovar K strain DK-CPA
avian serovar N strain FMN
avian serovar Q strain L3-10B
avian serovar R strain DRA-O
avian strain 700
avian strain 1220
avian strain 1223
avian strain 19756
bovine group 7 strain PG50

M.hyopneumoniae and *muris* which were obtained from Dr J.G. Tully, National Institutes of Health, Maryland, U.S.A. *M.dispar*, *M.feliminutum*, *M.flocculare*, *M.hyosynoviae* and *M.pneumoniae* were supplied by Dr R.H. Leach, Colindale, England. *M.hyopharyngis* and *M.mobile* were obtained from both Dr J.G. Tully and Dr R.H. Leach. In addition, *M.cavipharyngis*, *M.collis*, *M.cricetuli*, *M.felifaucium* and *M.testudinis* were obtained from Dr A. Hill, Carshalton, Surrey.

Three strains of mycoplasma isolated from geese (strains 1220, 1223 and 19756) were supplied by Dr Z. Varga, Hungarian Academy of Sciences, Budapest, Hungary, and one strain (700), recently isolated from a chicken in Spain, was obtained from Dr D. Bencina, University of Ljubljana, Yugoslavia. These strains each represented potential new species of *Mycoplasma*.

Antisera

Antisera prepared in rabbits against the mycoplasma species given in Table 2:1 were supplied by the FAO/WHO Collaborating Centre for Animal Mycoplasmas, University of Aarhus, Denmark, with the exception of antisera against M.*ellychniae*, *M.fastidiosum*, *M.genitalium*, *M.hyopharyngis*, *M.mobile* and *M.muris* which were obtained from Dr J.G. Tully. Antisera to *M.cavipharyngis*, *M.collis*, *M.cricetuli*, *M.felifaucium* and *M. testudinis* were obtained from Dr A. Hill. Antisera had been prepared in rabbits in our laboratory against strains 1220, 1223, 19756 and 700.

2.8 Viable counts

The technique used to determine the number of viable mycoplasmas was based on the method of Miles *et al.* (1938) as modified by Bradbury and Jordan (1971a).

Serial tenfold dilutions from 10^{-1} to 10^{-6} were made from the mycoplasma culture in MB using an electro-digital pipette[1] with disposable tips. Each dilution was mixed for 10 seconds on a vortex mixer[2] before 0.1 ml of the mixture was transferred using a new tip into 0.9 ml of MB. This step was repeated until all the required dilutions were prepared. Each dilution, as well as the undiluted original culture, was re-mixed on the vortex mixer and then, using an Eppendorf pipette[3] with disposable tips, five 0.02 ml drops were plated out with the minimum of delay onto a pre-dried plate containing 7 ml of MA. The plates were left for half an hour until the drops were absorbed and then incubated in a candle jar at 37°C for up to 7 days until the colonies were large enough to be counted. Dilutions whose colony counts fell between the range of 30-300 per drop were considered acceptable. The number of viable organisms in 1 ml of the original culture was calculated assuming that each viable 'unit' gave rise to one colony. Results are expressed as colony-forming units/ml (CFU/ml).

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1 Rainin Research Group, Taiwan

2 Stuart Scientific Co. Ltd., U.K.

3 Eppendorf Geratebau, Netheler + Hinze GmbH, Hamburg,.Germany

Chapter 3 Comparison of strains 4229 and B2/85 by restriction endonuclease analysis of their DNA

Chapter 3 Comparison of strains 4229 and B2/85 by restriction endonuclease analysis of their DNA

3.1 Introduction

Application of restriction endonucleases that recognize specific nucleotide sequences of DNA molecules has proved to be a useful and powerful method for determination of genetic relatedness among mycoplasma strains (Razin *et al.*,1983a, b). The method is used for comparing and differentiating between strains within mollicute species by analysing the DNA 'fingerprints' resulting from hydrolysis by restriction endonuclease enzymes, and separation of the resulting fragments by gel electrophoresis.

For example genetic homogeneity of five *M.pneumoniae* strains was demonstrated by Chandler *et al.* (1982) using this technique. It was demonstrated that there was marked genotypic heterogeneity among strains of *Acholeplasma axanthum* and less marked heterogeneity for *Acholeplasma oculi* strains, while a high degree of genetic homogeneity within strains of *Mycoplasma genitalium* was observed (Razin *et al.*, 1983b). Marked heterogeneity was also observed by Mew *et al.* (1985) in the cleavage patterns of digested DNA of 16 isolates of *M.ovipneumoniae* . In addition, genetic heterogeneity was found in the DNA cleavage patterns of *M.gallisepticum* strains (Razin *et al.*, 1983b; Santha *et al.*, 1988).

The technique has been used by other workers (Kleven *et al.*,1988a, b; Yogev *et al.*, 1988a) and has been found to be a useful tool for epidemiological studies for comparing and differentiating both *M.gallisepticum* and *M.synoviae* strains (Kleven *et al.*, 1988a; Morrow *et al.*, 1990).

The vaccine strain (F-strain) has been differentiated from other strains of *M.gallisepticum* by their DNA cleavage patterns (Khan and Yamamoto, 1989). These workers found that restriction endonuclease analysis of the DNA was more sensitive for strain differentiation of *M.gallisepticum* than analysis of the protein profiles by SDS-PAGE.

In view of the apparent similarities between strain 4229, the duck isolate from France, and strain B2/85, the isolate from a partridge in England, it was decided to examine their genotypic relatedness by comparison of their DNA cleavage patterns.

3.2 Materials and Methods

The method used was basically as described by Kleven *et al.* (1988a) with some modifications.

3.2.1 Preparation of mycoplasma DNA

Materials:

Tris-EDTA (washing buffer)

(10mM Tris HCl plus 1mM ethylenediaminetetraacetate)

To prepare 1 litre:

Tris HCl	1.58 g
EDTA[1]	0.37 g
Deionised, distilled water (DDW)	1000.0 ml

The pH was adjusted to 7.8-8.0.

Buffered phenol (phenol-TE buffer)

10.0 g of phenol powder[1] were dissolved in 10 ml of Tris-EDTA and incubated at 56°C for 5 minutes. The solution was shaken vigorously until emulsified and was then centrifuged at 1,000 g for 10 minutes. Using a sterile pipette, the upper aqueous layer was discarded. The above step was repeated twice. The resulting non-aqueous layer was stored at 4°C.

Phenol-chloroform solution

A chloroform:isoamyl-alcohol mixture was prepared in the ratio of 24:1 (v:v). The buffered phenol chloroform mixture was made by mixing equal volumes of this with phenol-TE-buffer. It was stored at 4°C.

Sodium dodecyl sulphate (SDS)

A 10% (w/v) solution was prepared in sterile distilled water in a fume cupboard.

Proteinase K

A stock solution of 10 µg per ml of proteinase[1] was used.

Ribonuclease (RNase)

A stock reagent of 100,000 Units (1 ml) from *Aspergillus oryzae*[2] was used.

Mycoplasma strains

Strains 4229, B2/85, *M.gallisepticum* S6LP and the type strain PG31 were used. All had been cloned three times (2.6) and their identity confirmed by indirect immunofluorescence.

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1 Sigma, Chemical Company, St.Louis, U.S.A.

2 Pharmacia, U.S.A.

Procedure:

Growth and preparation of organisms

The mycoplasmas were propagated in approximately 150 ml MB at 37°C until the phenol red indicator changed to an orange colour. They were harvested by centrifugation[1] at 2,200 g for 90 minutes at 4°C in sterile tubes. The supernatants were removed immediately, and the pellets suspended in 1.5 ml of Tris-EDTA and transferred to sterile microcentrifuge tubes[2]. They were washed three times in Tris-EDTA with centrifugation at 12,500 g in a Microspin 24 centrifuge[3]. The washed pellets were then resuspended in 0.6 ml of Tris-EDTA and transferred to sterile microcentrifuge tubes.

Cellular lysis

The mycoplasmas, suspended in the Tris-EDTA, were lysed by adding 100 µl SDS solution and 7 µl of proteinase K and incubating overnight at 37°C in a water bath.

Elimination of RNA

The RNase was added (5 µl) and the mixture was replaced in the 37°C water bath for 30 minutes.

Deproteinization

Phenol chloroform solutions were used to denature the proteins of the organisms leaving the nucleic acids in solution in an upper aqueous layer.

The lysed mycoplasma sample was extracted as follows:

---

1 Mistral 3000, MSE Scientific Instruments, Crawley, Sussex, U.K.

2 Sarstedt, W.Germany

3 Sorvall Instruments, Dupont Co. U.S.A.

1. A 750 µl aliquot of phenol-TE buffer was added to the sample and thoroughly mixed until an emulsion formed. It was then centrifuged for 5 minutes at 12,500 g and the emulsion separated into two distinct layers. The upper aqueous layer containing the nucleic acid was carefully removed, avoiding the interface layer, and transferred to a clean tube. The above step was repeated once.

2. A 750 µl aliquot of phenol-chloroform solution was added to the aqueous layer containing the crude DNA, mixed thoroughly, centrifuged at 12,500 g for 5 minutes and the resulting aqueous layer removed to a fresh tube. This procedure was repeated once.

3. A 750 µl volume of chloroform:isoamyl alcohol solution was added, mixed thoroughly, then centrifuged for 5 minutes and the resulting aqueous layer was removed to a fresh microcentrifuge tube. This step is used to remove the phenol.

Precipitation of DNA

The sample volume was measured and 1/10 (v/v) of 3M sodium acetate (pH 5.0) was added. Then 2 volumes of prechilled 95% (v/v) ethanol were added to one volume of sample. The mixture was shaken gently and kept at -20°C overnight. The precipitated DNA was collected by centrifugation at 12,500 g for 10 minutes. The supernatant was poured off and the pelleted DNA resuspended in 50 µl Tris-EDTA and dissolved by placing in a water bath at 37°C for 5-10 minutes. The DNA solution was stored at -20°C.

3.2.2 Digestion of DNA with restriction endonuclease enzymes

Materials:

Endonuclease enzymes

Three enzymes were used:

*Eco*RI[1], *Hin*dIII[1] and *Bgl*II[2]. The stock concentration of the first two was 20 unit/µl and of *Bgl*II was 10 unit/µl

Stop buffer

To prepare 100 ml:

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Glycerol	30.0 ml
Distilled deionised water	70.0 ml

Procedure:

This was based on the method described by Kleven *et al.* (1988a). A volume of 12.0 µl of purified DNA was digested with 2 µl of the appropriate restriction endonuclease and 3 µl of digestion buffer (supplied by the manufacturer) and the total volume of the mixture was made up to 30 µl by addition of deionized water. The mixture was incubated at 37°C for 1-2 hours and the reaction was stopped by addition of 5 µl of the stop buffer.

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1 Sigma Chemical Company, St Louis, MO, U.S.A

2 Pharmacia LKB Biotechnology, Uppsala, Sweden

3.2.3 Electrophoresis of digested DNA

Materials:

Phage lambda (λ) DNA *Hin*dIII digest

This DNA[1] was used as a molecular weight marker. The stock concentration was 300 µg/ml.

TBE buffer (running buffer)

To prepare 1 litre of concentrated stock buffer solution (10X) Sambrook *et al.* (1989):

Tris base (Trizma)[1]	109.0 g
Boric acid[1]	55.0 g
EDTA (Ethylene-diaminetetra-acetate)	9.5 g
DDW	1000.0 ml

The pH was adjusted to 7.5-7.8.

A working solution of 1X for agarose gel electrophoresis was prepared by 1:10 dilution of the above in DDW. The concentrated stock solution tended to develop a precipitate on storage. It was kept in glass bottles at room temperature and any batches with precipitate were discarded.

Agarose gel

A 0.8% solution of agarose[1] was prepared by dissolving 2.0 g in 250 ml of 1X TBE in a boiling water bath. The agarose solution was transferred to a water bath at 56°C for 20-30 minutes and then left at room temperature for 10-15 minutes. The agarose was poured into the electrophoresis tray to give a gel of 5 mm depth. A gel comb of 1

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[1] Sigma Chemical Company, St Louis, MO, U.S.A

cm slot size was placed in the gel which was left to solidify, and then transferred to a refrigerator for 15 minutes. The gel was carefully immersed into TBE after removing the comb and, just before adding the digested DNA, the volume of TBE was lowered until it was level with the gel surface.

Ethidium bromide solution

A 10 mg/ml solution of ethidium bromide[1] was prepared in distilled water and stored at room temperature in the dark.

Procedure:

Digested DNA was added to the wells in the gel and the phage DNA marker was included on each run. Electrophoresis was started at 100 V for 15-20 minutes, then continued at 30 V for 18-21 hours. The gel was gently washed with tap water, stained with ethidium bromide solution for 15-20 minutes and washed in tap water for 20-30 minutes. It was examined under UV illumination[2] and photographed with a Polaroid camera[3] using film 655[3].

3.3 Results

The restriction enzyme cleavage patterns of the DNA of strains 4229, B2/85, *M.gallisepticum* PG31 and S6 digested by *Eco*RI, *Hin*dIII and *Bgl*II are given in Figure 3:1A, B & C respectively.

Approximately 40 bands were seen in the *Eco*RI digests and about 25 in the *Hin*dIIII digests. The *Bgl*II cleavage patterns contained approximately 30 bands.

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1 Sigma Chemical Company, St Louis, MO, U.S.A.

2 LKB-Produkter AB, Bromma, Sweden

3 Polaroid Corporation, Cambridge, U.S.A.

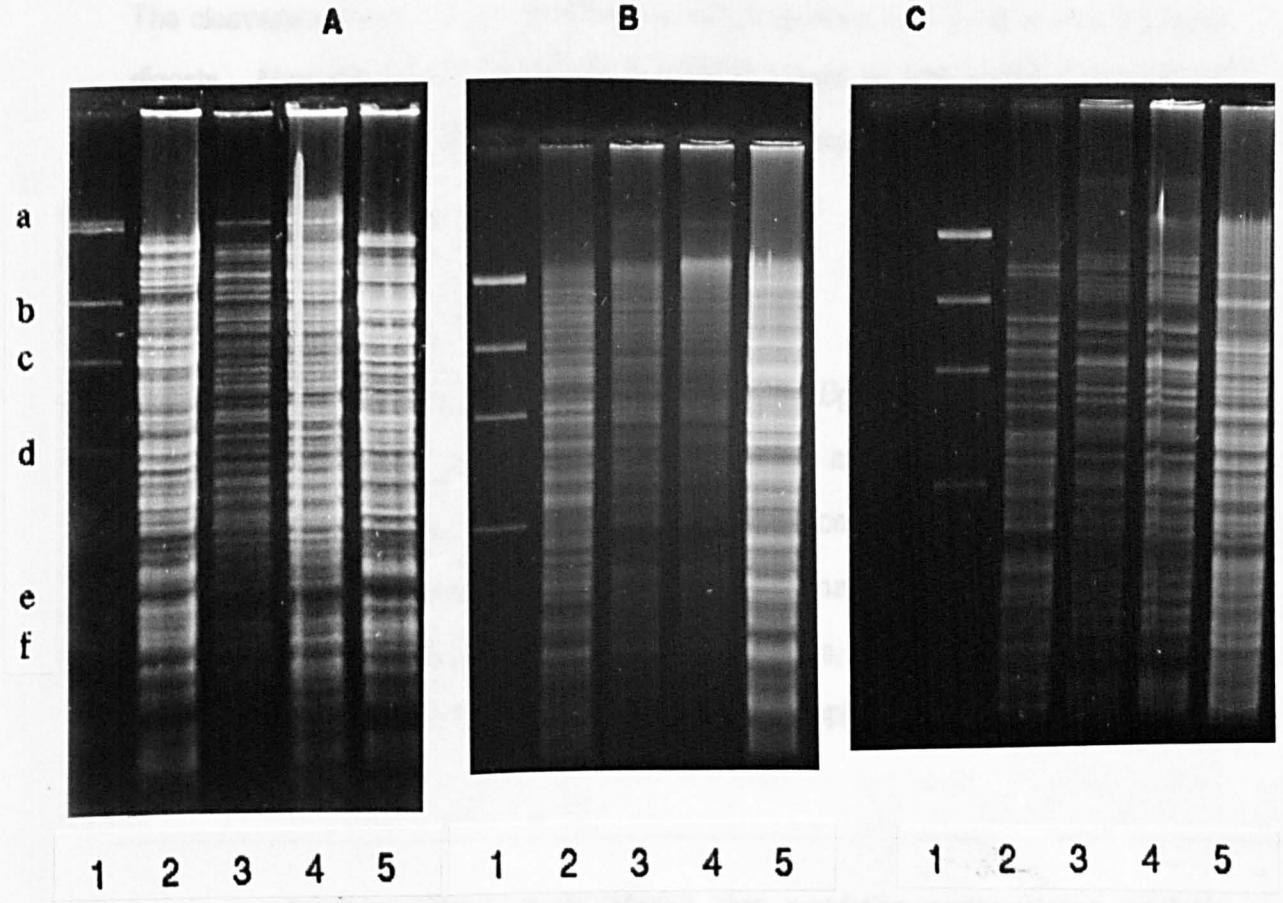

Fig. 3:1 Restriction endonuclease cleavage patterns of strains 4229, B2/85, *M.gallisepticum* PG31 and S6 digested with *Eco*RI (A), *Hin*dIII (B) and *Bgl*II (C). Lane 1 = phage lambda *Hin*dIII digest; 2 = PG31; 3 = 4229; 4 = B2/85; 5 = S6. Lambda molecular weight markers: a = 23.1; b = 9.4; c = 6.6; d = 4.4; e = 2.3; f = 2.0 KDa.

The cleavage patterns of strains 4229 and B2/85 appeared to be identical in all three digests. *M.gallisepticum* strains S6 and PG31 could be differentiated from each other and from strains 4229 and B2/85 with each of enzymes used.

3.4 Discussion

Using the restriction enzymes *Eco*RI, *Hin*dIII and *Bgl*II no differences could be detected between strains 4229 and B2/85 but they appeared different to the two strains of *M.gallisepticum*. These enzymes were chosen because they have been found to give many cuts in the DNA of mycoplasmas and thus have a greater tendency to emphasise the differences between strains, whereas those enzymes that give fewer cuts, such as *Bam*HI, *Kpn*I and *Xho*I, emphasise similarities (Kleven *et al.*, 1988a).

It was suggested by Kleven *et al.* (1988a) that restriction endonuclease analysis would have limited application for speciating unknown avian *Mycoplasma* isolates because of the variation that can occur between strains within a species. They found dissimilar patterns were given by numerous strains of *M.gallisepticum* after digestion with *Eco*RI, *Hin*dIII or *Bgl*II, although identical patterns were seen with all the strains when *Bam*HI and *Kpn*I were used (Kleven *et al.*, 1988a, b). Therefore the technique appeared to have more use for differentiation between strains within a *Mycoplasma* species than for speciation of unknown strains. Thus in the present studies it appears that strains 4229 and B2/85 are identical or very similar to one another, and different to the two strains of *M.gallisepticum*, but it is not possible with this technique to determine whether or not they belong to the species *M.gallisepticum*.

Our findings with strains 4229 and the two strains of *M.gallisepticum* are in agreement with the earlier studies by Dupiellet (1988) and Dupiellet *et al.* (1990) when they used the restriction enzymes *Eco*RI, *Hin*dIII and *Bam*HI to show

differences between their cleavage patterns. They also included a goose strain (35105) and found it to be identical to strain 4229. Like Khan and Yamamoto (1989) they also preferred restriction enzyme analysis of the DNA to SDS-PAGE of the cell proteins for distinguishing strains, although their cell protein profiles also showed that the duck and goose strains were similar to one another and different to *M.gallisepticum* strains PG31 and S6.

DNA:DNA hybridization studies performed by Dupiellet (1988) and Dupiellet *et al.* (1990) suggested a very high level of homology (95.7%) between the duck (4229) and goose (35105) strain but a much lower homology (approximately 40%) between these strains and *M.gallisepticum* PG31 and S6. It was this difference that prompted the suggestion that the duck and goose strains belong to a species distinct from *M.gallisepticum* and therefore might well represent a new species (Dupiellet *et al.*, 1990).

Johnson (1984) considered that organisms with a DNA homology of between 60 and 100% could be considered to belong to the same species and those with homologies between 20 and 60% to represent closely related species. Athough these criteria are arbitrary, they support the suggestion that the duck and goose strains are not *M.gallisepticum* but belong to a closely related species. This view is reinforced by the suggestion of the *Ad Hoc* Committee of the ICSB (1987) that the phylogenetic definition of a species would include strains with approximately 70% or greater DNA:DNA relatedness.

Similarities between the duck and goose strains, and differences between these and *M.gallisepticum* were also seen when the strains were analysed using two different rRNA gene probes (Dupiellet, 1988). As mentioned in Chapter 1 these findings were supported by those of Yogev *et al.* (1988a) who used the rRNA gene probe pMC5 (Amikam *et al.*, 1982) and demonstrated the similarity of strain 4229, a goose strain

(30902) and our strain B2/85. These three strains showed a unique fingerprint that was different to those of *M.gallisepticum* strains. Similar results were obtained using a *tuf* gene probe (Yogev, 1988b). (At the time of their studies it was believed that all the strains were *M.gallisepticum*).

The molecular approach has shown that strain 4229 and related organisms can be considered as a species distinct from *M.gallisepticum* but, in order to propose that they represent a new species it is necessary to show that the organism is serologically distinct from all the recognised avian and mammalian *Mycoplasma* species and to describe its properties (Subcommittee on the Taxonomy of Mollicutes, 1979). The following Chapters provide a description of these studies.

Chapter 4 Morphological studies

Chapter 4 Morphological studies

4.1 Introduction

The classification and establishment of an organism within the class *Mollicutes* depends basically on the following criteria: absence of a cell wall and wall precursors, presence of a plasma membrane, absence of reversion to a wall-covered bacterium under appropriate growth conditions, filterability through a membrane filter of 450 nm pore diameter and production of minute colonies with typical 'fried-egg' appearance in almost all species. The organisms are pleomorphic, varying in shape from spherical or pear-shaped structures to branched or helical filaments (Subcommittee on the Taxonomy of *Mollicutes*, 1979; Freundt, 1983b).

The lack of a cell wall and presence of a triple-layered 'unit membrane' are the most prominent features that can be demonstrated by electron microscopy of the sectioned organisms to establish them as members of the class *Mollicutes* (Freundt and Edward, 1979). Lack of a cell wall is responsible for the characteristic colonial morphology, which is similar to that of the L-phase variants of bacteria (Neimark, 1979). Colonies produced by L-phase variants share the 'fried-egg' shape with mycoplasma colonies although the former usually appear coarser because they are made up of larger elements (Razin,1983a). The L-phase variants are produced in media containing antibiotics and certain other substances (Dienes and Weinberger, 1951) and thus, in order to prove that a new isolate belongs to class *Mollicutes*, it should be demonstrated that it does not revert to parent bacterial forms in media devoid of such substances (Subcommittee on the Taxonomy of *Mollicutes*, 1979). In addition, the possibility of the organisms being L-phase variants can be excluded by demonstrating their inability to synthesise the basic constituents of the bacterial cell wall including the muramic acid and diaminopimelic acid (Freundt *et al.*,1979).

The characteristic morphology of the mycoplasma organisms (members of the family *Mycoplasmataceae* and *Acholeplasmataceae*) has been described as usually pleomorphic and consisting of small coccoid, ring forms and fine branched filaments of varying length, while the morphology of other organisms (members of the family *Spiroplasmataceae*) has been described as helical in most circumstances (Subcommittee on the Taxonomy of *Mollicutes*, 1979). Helical morphology and motility in spiroplasmas has been demonstrated by dark-field microscopy in broth medium, but non-motile, non-helical filaments and spherical bodies are seen in agar cultures (Cole, *et al.*, 1973). Mycoplasma morphology obtained with light microscopy techniques depends upon nutritional quality of the growth medium and its osmotic pressure, the phase of the growth culture (age of the culture) (Razin, 1983b) and the viability of the organisms (Bredt, 1983; Tully, 1983b).

Plasticity and small size are other important characteristics of the *Mollicutes* and have been determined by filterability through membrane filters of 200-450 nm pore diameter (Freundt and Edward, 1979). Most organisms of the class *Mollicutes* pass through a filter of 450 nm pore diameter and some may pass through pores of 220 nm diameter (Subcommittee on the Taxonomy of *Mollicutes*, 1979). The diameter of the coccoid bodies varies between 0.3-0.8 µm whereas the diameter of mycoplasma filaments is between 0.2-0.4 µm. The filaments may reach lengths of more than 100 µm and they may have a branched form. Filaments of helical morphology have a diameter of 80-200 nm and a length of 2-5 µm (Razin,1983b). Although organisms of less than 300 nm diameter (100 to 250 nm) have been reported and described as 'elementary bodies', they proved non-reproductive after filtration through a 220 nm pore membrane (Maniloff and Morowitz, 1972; Robertson *et al.*, 1975a).

The studies in this Chapter were undertaken to establish that strains 4229 and B2/85 belong to the class *Mollicutes*, and that they are differentiated from the family *Spiroplasmataceae* .

4.2 Materials and methods

4.2.1 Colony morphology

In order to demonstrate the ability of the untyped mycoplasma strains to produce typical 'fried-egg' shaped colonies, they were grown on MA and examined under a dissecting stereo-microscope[1]. Colonies of *M.gallisepticum* S6LP were used for comparison.

Procedure:

Serial tenfold dilutions to 10^{-3} were made from stock frozen cultures of strains 4229 and B2/85 and were inoculated onto 8 ml MA plates and incubated at 37°C for up to 7 days. The plates were examined daily at magnifications of x10 to x40, and the colonies were observed and photographed.

4.2.2 Electron microscopy

Electron microscopy was used to examine ultra-thin sections of strains 4229 and B2/85 for lack of a cell wall and for the presence of a triple-layered membrane. It was also of interest to look for the possible presence of a specialized attachment terminal structure as is seen in the closely related *M.gallisepticum* (Maniloff, *et al.*, 1965). The technique used was basically that described by Cole (1983) but with slight modifications.

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1 Olympus Optical CO. Ltd., Ogawamachi Kanda Chiyoda-Ku, Tokyo, Japan.

To avoid distortion of the mycoplasmas as much as possible broth cultures were harvested before pH change in the medium became obvious (i.e. when the phenol red indicator had just become orange), and a low speed of centrifugation was used for harvesting. Fixation of the cell pellets was with glutaraldehyde followed by post-fixation with osmium tetroxide. The latter fixative was used because the mycoplasma cell structure may be fixed with glutaraldehyde but may still be prone to distortion due to osmotic pressure and to drying (Boatman,1979). The fixed pellets were either embedded in agar and cut into cubes for ease of handling before post-fixation with osmium tetroxide, or they were suspended in the osmium tetroxide before they were embedded in the agar, and then cut into cubes.

Materials:

Veronal acetate buffer

Sodium veronal[1]	2.94 g
Sodium acetate	1.94 g
Sodium chloride	3.40 g
Distilled water	100.0 ml

Kellenberger buffer (Hayat, 1972)

Veronal acetate buffer	5.0 ml
Hydrochloric acid (0.1 M)	7.0 ml
Calcium chloride dihydrate (1.0 M)	0.25 ml
Distilled water	13.0 ml

The pH was adjusted to 6.0 using 0.1 M hydrochloric acid. Freshly prepared buffer was used.

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1 Taab Laboratories Equipment Ltd., Reading, Berkshire, U.K..

Primary fixative (2.5% glutaraldehyde)

Glutaraldehyde(25%) [1]	1.0 ml
Mycoplasma broth	9.0 ml

The above ingredients were mixed and stored at 4°C and fresh fixative was prepared for each experiment.

Secondary fixative (1% osmium tetroxide solution)

Osmium tetroxide[2]	1.0 g
Veronal acetate buffer	100.0 ml

The osmium tetroxide was supplied in a glass ampoule and was opened in a fume cupboard inside a glass bottle to avoid handling. The buffer was then added and left at 4°C for 1-2 days for the osmium tetroxide to dissolve. The glass fragments were removed and the solution was stored at 4°C in the dark. It was discarded when a deep straw colour developed.

Standard embedding medium (Spurr, 1969)

ERL 4206 (vinyl cyclohexene dioxide) (Epoxy resin)[3]	10.0 g
D.E.R. 736 (diglycidyl ether of polypropylene glycol)[3]	6.0 g
Nonenyl succinic anhydride (NSA)[3]	26.0 g
S-1 or dimethylaminoethanol (DMAE)[3]	0.4 g

1 Taab Laboratories Equipment Ltd., Reading, Berkshire, U.K.
2 Johnson Matthey Chemicals Ltd., Royston, Hertfordshire, U.K.
3 Bio-Rad Microscience Division, Watford Business Park, U.K.

The above ingredients were mixed in a polyethylene bottle in the order shown above and the medium was stored at -20°C.

Stains

Lead citrate

Solution A	
Lead nitrate EM[1]	1.33g
Tri-sodium citrate[2]	1.76 g
Distilled water	30.0 ml
Solution B	
Sodium hydroxide (1.0 M)	8.0 ml
Distilled water	

The solid ingredients in solution A were added to the distilled water and shaken vigorously for 1 minute, then allowed to stand for 30 minutes with intermittent shaking to complete conversion of the lead nitrate to lead citrate. Then solution B was mixed with solution A and the volume was made up to 50 ml with distilled water. The mixture was filtered to remove precipitate and stored at 4°C, It was discarded if turbidity or precipitation developed.

Uranyl acetate

Uranyl acetate EM[1]	0.5 g
Distilled water	100.0 ml

The uranyl acetate was dissolved in distilled water, filtered to remove undissolved particles and stored at 4°C. It was also filtered immediately before use.

1 Taab Laboratories Equipment Ltd., Reading, Berkshire, U.K.
2 BDH Chemicals Ltd., Poole, Dorset, U.K.

Procedure:

The mycoplasma strains were grown in 200 ml MB at 37°C for 24-48 hours (depending on the strain) until the colour of the phenol red indicator started to change. The cultures were harvested by centrifugation at 2,000 g for 10 minutes The supernatant was removed and any last few drops of moisture were dried with filter paper strips. Using a sterile Pasteur pipette each pellet was gently suspended in 5 ml of 2.5% glutaraldehyde in mycoplasma broth (primary fixation) and allowed to fix for 2-4 hours at room temperature. For each strain the suspension was now divided into two parts, A and B, and centrifuged at 2,000 g for 5 minutes. Each pellet was washed three times by gentle resuspension in 2 ml Kellenberger buffer followed by centrifugation at 2,000 g for 5 minutes. The supernatants were removed and the last few drops of moisture dried with filter paper strips. A 2% (v/v) solution of Noble agar[1] was made up in the Kellenberger buffer, well dissolved and cooled to 45°C. The pellets were then treated as A or B described below:

A. The pellets designated A were mixed quickly with 2-3 drops of the agar using a sterile warmed Pasteur pipette and then dispensed quickly onto alcohol-cleaned microscope slides. After solidification they were cut into 1 mm cubes. The cubes were suspended in 1% osmium tetroxide (secondary fixation) in veronal acetate buffer for 6 to 16 hours at room temperature. The cubes were transferred into veronal acetate buffer and washed for 15 to 20 minutes with frequent agitation.

B. The pellets designated B were gently resuspended in 1% osmium tetroxide for 6 to 16 hours at room temperature. They were centrifuged at 2,000 g for 5 minutes, washed once in veronal acetate buffer and then centrifuged again at 2,000 g for 5 minutes. The supernatant was removed and the last few drops of moisture were

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[1] Difco Laboratories Ltd., Detroit, Michigan, U.S.A.

dried with filter paper strips and then 1 mm cubes of agar-containing mycoplasmas were prepared by the procedure described for pellet A.

All agar cubes were suspended in 0.5% aqueous uranyl acetate for 16 hours at 4°C. They were dehydrated by transfer through a series of acetone[1] solutions of increasing concentration (70%, 90% and 100%) for two periods of 5 minutes at each concentration. The acetone in the last of the series was replaced with 1 ml of fresh acetone (100%) and 1 ml of Spurr's medium, and gently mixed. The specimens were kept in this solution for 30 minutes with occasional mixing, after which the mixture was replaced with 1 ml fresh acetone (100%) and 3 ml of Spurr's medium and allowed to stand for a further 30 minutes. The mixture was replaced with 2 ml of Spurr's medium and left for 1 to 2 hours until the cubes sank to the bottom of the container.

The cubes were placed in polythene embedding capsules[2] to which fresh Spurr's medium was added. They were allowed to stand for 1-2 hours to allow any air bubbles to rise to the surface. The caps of the capsules were removed and the capsules placed in an oven at 70°C for 24 hours for polymerisation.

Sections of 0.1 um thickness were cut with a glass knife on a Reichert Ultracut[2] and collected on 3 mm copper grids (200 mesh)[3]. They were stained with uranyl acetate and lead citrate. This was done by placing the grids containing the section over a drop of uranyl acetate fixed to a Parafilm strip placed inside a Petri dish. After 5 to 10 minutes the grids were rinsed by dipping in three changes of sterile distilled water for 20 seconds each time. The grids were dried by touching the edge with filter paper and then they were placed face downwards on a drop of lead citrate fixed over a strip

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1 May and Baker Ltd., Dagenham, Essex, U.K.
2 Reichert-Jung, Cambridge Instruments Ltd., U.K.
3 Taab Laboratories Equipment Ltd., Reading, Berkshire, U.K.

of Parafilm as above. After 5 to 10 minutes they were rinsed in three changes of sterile distilled water and dried as before. The grids were examined with an electron microscope [1] with an accelerating voltage of 80 Kv. Electron micrographs were taken using Ilford E. M. film[2].

4.2.3 Absence of reversion

In order to rule out the possibility that the untyped strains were L-phase variants of bacteria they were passaged in media devoid of bacterial inhibitors and examined for possible reversion. Broth medium is preferred for this examination (Subcommittee on the Taxonomy of *Mollicutes*, 1979).

Procedure:

Cultures of the lowest available *in vitro* passage level were prepared. Thus strain 4229 had undergone its seventh passage in our laboratory but its history before this was not available. Strain B2/85 was used after one cloning and the culture was of passage level seven. These cultures were then passed ten consecutive times in MB without penicillin or thallium acetate. At each passage, subculture was carried out onto blood agar and MA. Smears were made from each MB passage and from colonies which grew on the blood agar. They were stained with Giemsa and Gram stains and examined microscopically at a magnification of x1,250. The identity of the colonies which grew on MA after each passage was checked by indirect immunofluorescence (6.2.2).

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1 Hitachi H-600, Tokyo, Japan.
2 Ilford Ltd., U.K.

4.2.4 Light microscopy

Examination of broth cultures of organisms by dark-field and phase contrast microscopy to confirm their pleomorphic characteristic was carried out as recommended by the Subcommittee on the Taxonomy of *Mollicutes* (1979).

Procedure:

Strains 4229 and B2/85 were prepared after 24 and 72 hours incubation for examination by phase-contrast and dark-field microscopy according to method of Turner (1935). Both strains were grown in Seitz-filtered MB and a small drop of the culture was placed in the centre of a clean microscopic slide and then inverted over a coverslip positioned on a pad of filter paper. A second pad of filter paper was placed over the coverslip and pressed gently to obtain a thin film of the culture. The edge of the coverslip was sealed with molten paraffin wax and the slide examined under a Leitz Ortholux microscope[1] at magnification of x540 to x950.

4.2.5 Filtration studies

Determination of the filterability of strains 4229 and B2/85 was carried out using membrane filters of 450 nm and 220 nm pore diameter.

Procedure:

An overnight mycoplasma broth culture of each strain was diluted tenfold in PBS (pH 7.3) containing gelatin[2] (0.2% v/v). A 2 ml volume was taken into a disposable plastic syringe and attached to a Swinney-type adapter containing either a 450 nm or

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1 E.Leitz (Instruments) Ltd., London, U.K.
2 BDH Chemicals Ltd., Poole, Dorset, U.K.

a 220 nm pore diameter membrane filter[1] . Filtration was carried out with gentle pressure and viable counts (2.8) were determined before and after filtration.

4.3 Results

4.3.1 Colony morphology

Strains 4229 and B2/85 both produced typical 'fried-egg' colonies as demonstrated in Figures 4:1 and 4:2. The colonies of each strain had smooth entire edges with a small, darkly pigmented central zone and they were considerably larger than those of *M.gallisepticum* S6LP (Figure 4:3).

4.3.2 Electron microscopy

The results obtained for both untyped strains were the same whichever method of secondary fixation had been used (i.e. before or after the organisms were embedded in agar).

The two untyped strains possessed a triple-layered membrane and showed no evidence of a cell wall (Figures 4:4A & B, 4:5A & B). Almost all of the organisms of both strains were spherical, ovoid or pear-shaped. The ribosomes were distributed throughout the cytoplasm and areas of high electron density were observed near to the membrane.

The most prominent feature of both these organisms was that many of the cells possessed a tip-like structure at one end which was similar in appearance to the specialized bleb structure found in *M.gallisepticum* . At higher magnifications this

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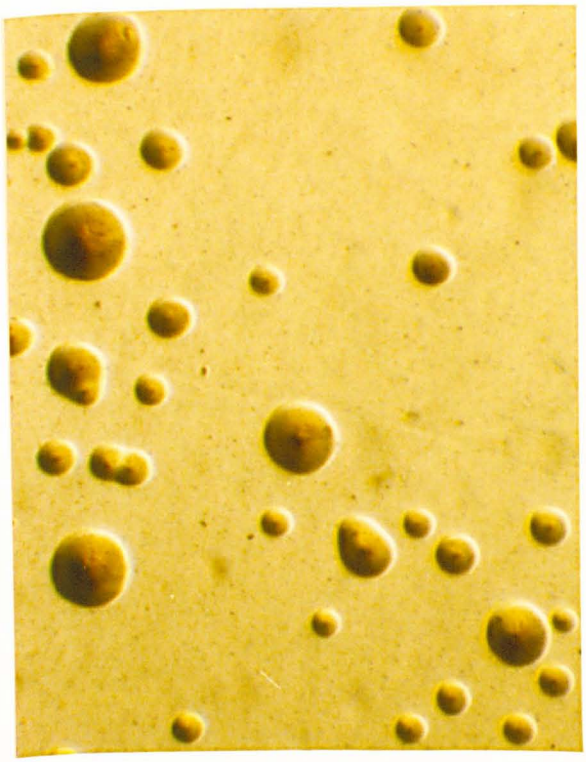
1 Sartorius, Minisart NML GmbH, D-3400 Gottingen, Germany.



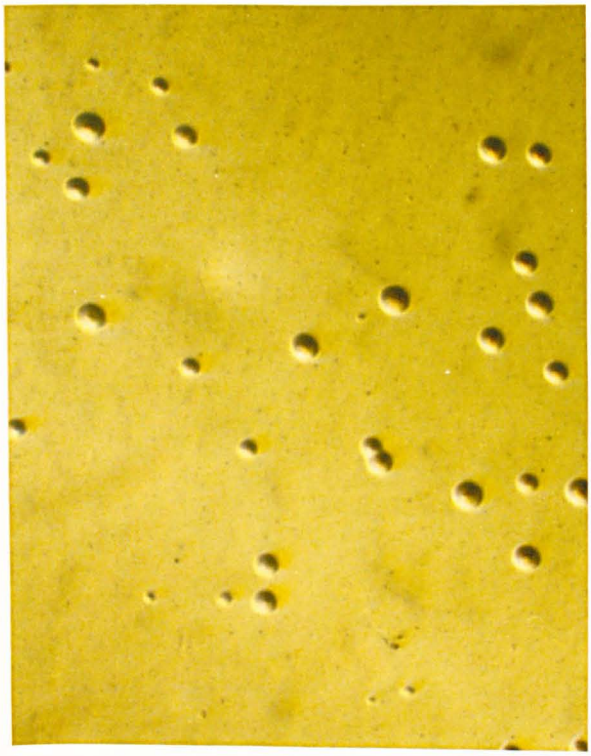
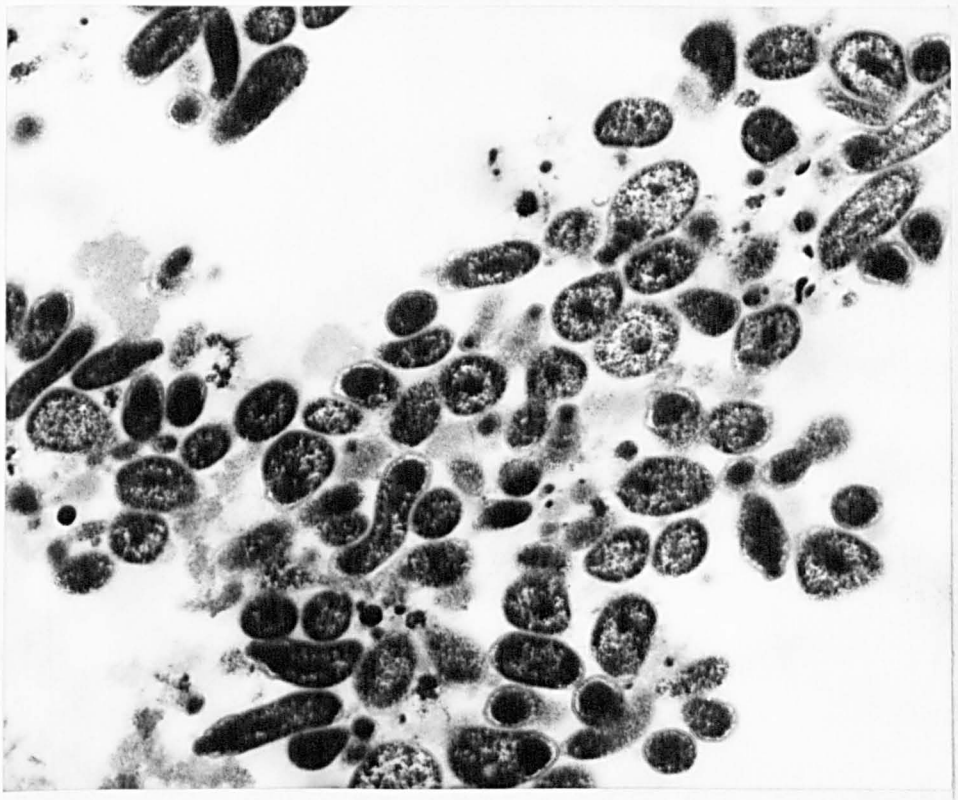


Fig. 4:1 Colonies of strain 4229 **Fig. 4:2 Colonies of strain B2/85**

Fig. 4:3 Colonies of *M.gallisepticum* S6LP

All colonies photographed after 48 hours incubation. x60

A



B

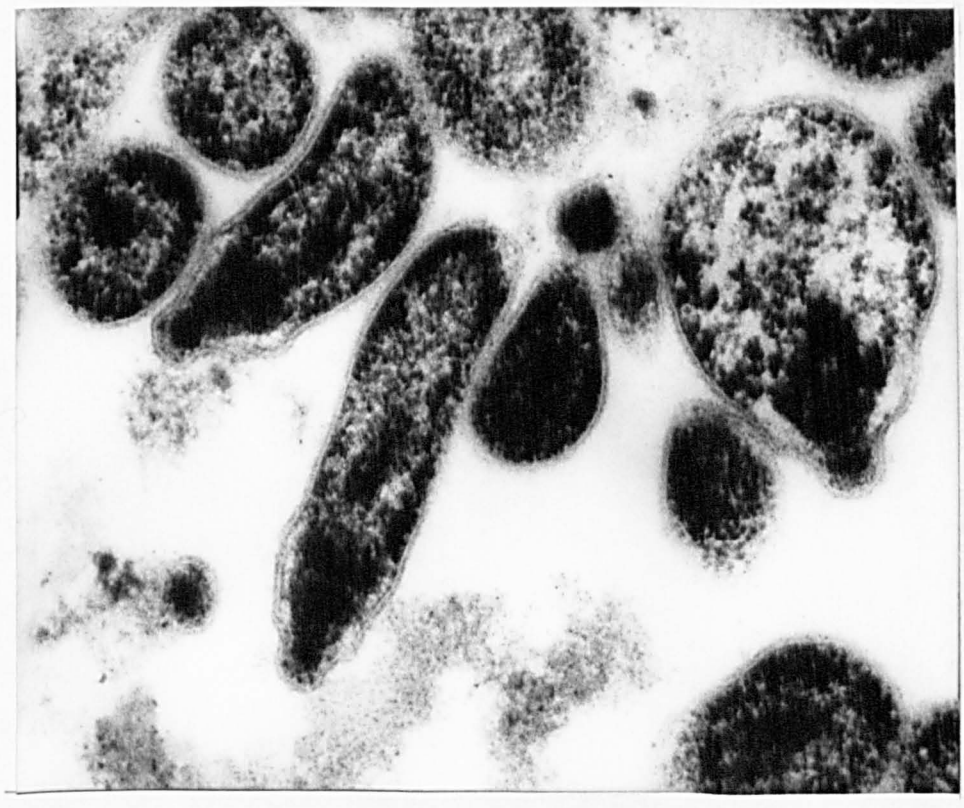
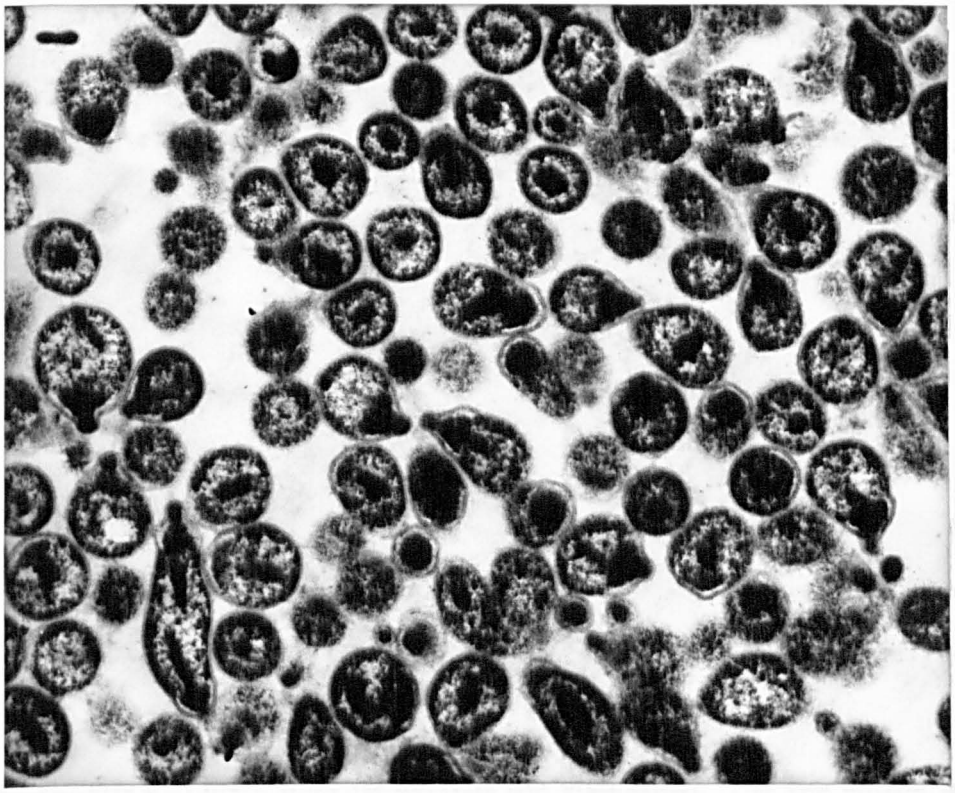


Fig. 4:4 Electron micrograph of strain 4229

A. Ultrathin section showing round, elongated and flask-shaped organisms. x18,000

B. Ultrathin section showing triple layered membrane and terminal tip organelle. x60,000

A



B

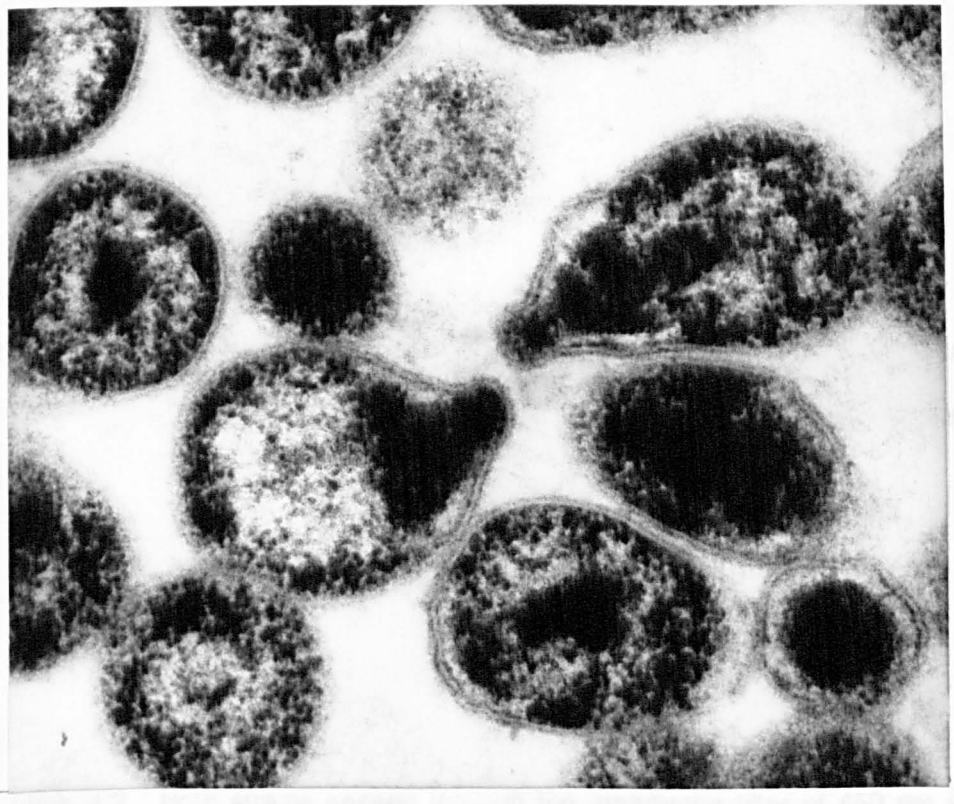


Fig. 4:5 Electron micrograph of strain B2/85

A. Ultrathin section showing round and flask-shaped organisms. x18,000

B. Ultrathin section showing triple layered membrane and terminal tip organelle. x60,000

structure appeared to contain an area of higher electron density and the membrane surrounding it was a continuation of the triple-layered membrane but with a higher intensity of staining.

4.3.3 Absence of reversion

There was no evidence of reversion of the organisms to bacterial forms after serial passage through broth medium without penicillin or thallium acetate. After each passage, characteristic colonies grew on subculture onto agar and their identity was confirmed. The Giemsa- and Gram-stained smears indicated the presence of mycoplasmas and that there were no bacteria-like organisms. The two strains were Gram negative organisms.

4.3.4 Light microscopy

The morphological forms of strains 4229 and B2/85 as seen by light microscopy are indicated in Table 4:1. In both strains the organisms appeared as coccoid, bacillary, ring or flask shapes at 24 and 72 hours. There was no indication of motility nor were any helical forms seen.

4.3.5 Filtration studies

The numbers of the viable organisms before and after passage through the filters are given in Table 4:2. Both strains passed through the membrane filter of 450 nm with reduction in titre of approximately10^2 and they passed through the membrane filter of 220 nm with reduction in titre of approximately 10^3.

Table 4:1 Morphology of strain 4229 and B2/85 by light microscopy

Strain	Morphological forms					
	Coccoid					
	Single/ double	Short chains	Clumps	Bacillary/ short filaments	Ring forms	Other forms
4229	+[1]	-[2]	+[3]	+	-	flask shaped
B2/85	+	-	+3	+[3]	+	flask shaped

1. Present
2. Absent
3. Present after 72 hours

Table 4:2 The viability of strains 4229 and B2/85 before and after filtration

Strain	Viable count (CFU/ml)		
	Pre-filtration	Post-filtration	
		450 nm	220 nm
4229	7.2×10^6	4.0×10^4	1.6×10^3
B2/85	6.7×10^6	9.9×10^4	3.6×10^3

4.4 Discussion

The duck and partridge strains, 4229 and B2/85, both developed the typical 'fried-egg' colonies indicating that they belong to the class *Mollicutes* (Subcommittee on the Taxonomy of the *Mollicutes*, 1979), but this characteristic is also shared with bacterial L-phase variants. The other features seen in these strains were the possession of a triple-layered membrane and absence of a cell wall which also indicate that they belong to the class *Mollicutes* but again does not rule out the possibility that they are bacterial L-phase variants.

The morphology of fixed mycoplasmas prepared for examination by electron microscopy has been found to be influenced by several important factors including the phase of growth and viability of the organisms at the time of harvest, whether or not the organisms are fixed prior to collection, the concentrations of the buffer-fixative used and the osmolality of the final solution (Boatman, 1979). The broth cultures of strains 4229 and B2/85 used in these studies were harvested when the colour of the pH indicator started to change in order to examine actively and uniformly growing cells and to avoid the pleomorphic forms that may appear with further incubation (Robertson *et al.*, 1975 a).

The harvests of the mycoplasma strains in the present study were obtained before fixation in order to obtain a good pellet of organisms for processing (Cole, 1983) although it has been found that fixation prior to centrifugation of the cells will lessen the morphological distortion that results from the preparative procedures (Robertson *et al.*, 1975 b). In addition, the broth cultures were harvested at low centrifugal force to avoid distortion and morphological variation that can result at this step of the procedure, even though it is recognised that the use of such low centrifugal force might not harvest representative mycoplasma cells in the culture (Robertson *et al.*, 1975 b).

With regard to the concentrations of the buffer-fixative used and the osmolality of the final solution, these factors were less critical here as the organisms were removed from the broth by centrifugation before fixation (Lemcke, 1972) and the pellets were suspended in fixative made up in the broth medium and not in buffer, which might alter the morphological features of some mycoplasmas (Cole, 1983). Suspension in 2.5% glutaraldehyde made up in fresh mycoplasma broth seems to be preferable in order to balance osmolality and/ or pH (Cole and Popkin, 1981).

The morphology of strains 4229 and B2/85, as observed by electron microscopy, indicated that there was no difference in method of preparation for embedding the pellets of fixed organisms in agar, either before or after post-fixation with secondary fixative, although the latter technique is preferred for ease of handling of the pellets. Both strains prepared by either method demonstrated a triple-layered membrane and lack of the cell wall. The membrane consisted of two dense layers separated by a less dense area.

A specialised terminal organelle structure was observed in both strains and was similar to those that have been described in other mycoplasmas such as *M.gallisepticum* (Maniloff *et al.*, 1965), *M.pneumoniae* (Biberfeld and Biberfeld, 1970), *M.genitalium* (Tully *et al.*, 1983), *M.alvi* (Gourlay *et al.*,1977) and *M.mobile* (Kirchhoff *et al.*, 1987). These terminal organelles, which appeared at one end of the cell, consisted of material of higher electron density, surrounded by a clear space, and covered with an outer membrane which was a continuation of the triple-layered membrane. In addition, a spherical region between the terminal organelle and the rest of the cell, similar to the infra-bleb regions found in *M.gallisepticum* (Maniloff *et al.*, 1965; Maniloff and Morowitz, 1967; Allen *et al.*, 1970), was observed in the untyped strains.

Although these terminal organelles have not yet been fully characterized chemically or functionally, data obtained mostly with *M.pneumoniae* and *M.gallisepticum* implicates them as attachment organelles, although the attachment process might not be their unique function (Razin, 1985).

The association of *M.gallisepticum* with adherence was first reported by Zucker-Franklin *et al.*, (1966), when they noted that the bleb was the contact site more frequently involved when *M.gallisepticum* adhered to leukocytes. The possible role of the bleb in adherence of *M.gallisepticum* was also suggested by Uppal and Chu, (1977) who demonstrated that the mycoplasma cells often appeared to be attached by their blebs to the surface of the tracheal epithelium of chickens. The attachment of *M.pneumoniae* organisms by their terminal tip structure to the surface of respiratory epithelial cells has been demonstrated in tracheal organ culture by Collier *et al.*, (1971) although evidence presented later has suggested attachment of *M.pneumoniae* to host cells with membrane sites other than the tip structure (Brunner *et al.*, 1979).

Further studies were carried out into the possible role in adherence of the terminal organelle of strains 4229 and B2/85 and are described in Chapter 7.

The absence of reversion to bacterial forms by both untyped strains indicated that they are not bacterial L-phase variants, although we were not able to obtain and test a low passage culture of strain 4229.

Light microscopy demonstrated the presence of different morphological forms, with coccoid forms as the most prominent, although others such as flask shapes were observed on further incubation (at 72 hours). This agrees with the findings of Robertson *et al.* (1975a) who demonstrated that the number of pleomorphic forms of *M.hominis* increased upon further incubation. A ring form or ghost-like appearance

was observed in strain B2/85 but not in strain 4229. There was no evidence of helical forms in either strain 4229 or B2/85.

In the filtration studies, both untyped strains passed through membrane filters of 450 and 220 nm pore diameters but with some reduction in the titre. All members of the class *Mollicutes* can be passaged through filters of 450 nm pore diameter and some of them may also pass through pores of 220 nm (Sub-committee on the Taxonomy of *Mollicutes*, 1979).

In summary, the duck and partridge strains (4229 and B2/85, respectively) lack a cell wall and are bounded by a characteristic triple-layered membrane. The organisms possess a terminal tip structure similar to that of *M.gallisepticum*. They are pleomorphic and do not demonstrate a helical motile form. They are of small size and pass through membrane filters of 450 and 220 nm pore diameter. They show typical 'fried-egg' colony morphology and show no reversion to a wall-covered bacterium. On the basis of the above results, the two strains can be assigned to the class *Mollicutes* but excluded from the family *Spiroplasmataceae* . The fact that they were isolated and subcultured under aerobic conditions also excludes them from the order *Anaeroplasmatales* . The following sections describe further biochemical and serological studies which were carried out to characterize these organisms.

Chapter 5 Biochemical characterization

Chapter 5 Biochemical characterization

5.1 Introduction

The studies of the untyped strains described in the previous Chapter established that they belong to the class *Mollicutes* but that they could not be assigned to the family *Spiroplasmataceae* on morphological grounds, nor could they be assigned to the order *Anaeroplasmatales* since they were not obligate anaerobes (Subcommittee on the Taxonomy of *Mollicutes*, 1979).

The investigations in this Chapter were carried out in order to assign strains 4229 and B2/85 to an order, family and genus, and to provide a full description of the proposed new species. Thus direct and indirect methods were used to distinguish between the sterol dependent order, *Mycoplasmatales* and the non-sterol dependent *Acholeplasmatales* (Subcommittee on the Taxonomy of *Mollicutes*, 1979; Freundt, 1983b).

The family *Mycoplasmataceae* includes two genera, *Mycoplasma* and *Ureaplasma*, and differentiation between them depends on ability of the members of latter to hydrolyse urea with resulting production of ammonia and carbon dioxide (Shepard *et al.*, 1974; Freundt, 1983b). This property was investigated for strains 4229 and B2/85

Other biochemical tests were carried out to provide the description of the proposed species (Subcommittee on the Taxonomy of *Mollicutes*, 1979) although evidence that the organism represents a new species finally depends upon the serological methods described in Chapter 6.

5.2 Materials and methods

5.2.1 Sterol requirement

Sterol requirement of strains 4229 and B2/85 was investigated by direct assessment of a quantitative growth response of the organisms to increasing levels of cholesterol, and by indirect methods to measure the sensitivity of the organisms to digitonin and to sodium polyanethol sulphonate (SPS). Two methods of direct assessment of growth response to cholesterol were attempted. The first was in agar media (Edward, 1971) and the second in broth (Razin and Tully, 1970).

Sterol requirement using agar media

Materials:

Washing medium

Part A	
Bacto PPLO broth (w/o crystal violet)	1.47 g
Distilled water	78.5 ml
Part B	
Yeast extract (25% w/v)	10.0 ml
Bovine albumin fraction V[1] (10% w/v)	10.0 ml
Thallium acetate (5% w/v)	1.0 ml
Benzyl penicillin (200,000 i.u/ml)	0.5 ml

The ingredients of part A were autoclaved at 15 lb/sq.in for 15 minutes, allowed to cool and mixed with the sterile ingredients of part B.

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1 Sigma Chemical Co Ltd., St Louis, MO, U.S.A.

Basal medium (BMA)

Part A	
Bacto-PPLO (w/o crystal violet)	1.47 g
Lab M agar No.2	1.0 g
Distilled water	88.5 ml
Part B	
Yeast extract (25% w/v)	10.0 ml
Benzyl penicillin (200,000 i.u/ml)	0.5 ml
Thallium acetate (5% w/v)	1.0 ml

The ingredients of part A were autoclaved at 15 lb/sq.in for 15 minutes and allowed to cool to 56°C. The sterile ingredients of part B were warmed to 56°C and mixed aseptically with part A. Part B was supplemented with various additives and, accordingly, the proportion of the water in Part A was altered to maintain the concentration of other ingredients. These supplements are listed below.

Bovine serum albumin fraction V[1] (BSA)

A stock solution of 10% (w/v) was prepared in distilled water and the pH was adjusted to 7.5 with 1 M sodium hydroxide. It was sterilized by Seitz filtration and stored at 4°C.

Palmitic acid [1]

A stock solution of 10 mg/ml was prepared in 95% ethanol which was warmed gently to dissolve the fatty acid.

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1 Sigma Chemical Co. Ltd., St Louis, MO, U.S.A.

Cholesterol suspension

Stock solution of 1 mg/ml was prepared by dissolving 100 mg of cholesterol (either Sigma C8378[1], Koch-Light U.S.P[2] or Sigma cell culture-tested) in 2 ml of warm ethanol (95%) and then ejecting the solution into 100 ml distilled water, pH 7.0 at 80°C, using a Pasteur pipette. The ethanolic solution was kept warm and was introduced below the surface of the water and the pipette was pre-warmed to avoid crystallization.

The cholesterol test agars

The following 8 different media were prepared with addition of the above supplements:

1. Basal medium (BMA).
2. BMA with 0.5% (w/v) BSA and 10 ug/ml palmitic acid.
3. BMA with 0.5% (w/v) BSA and 10 ug/ml palmitic acid and 0.2 ug/ml cholesterol.
4. BMA with 0.5% (w/v) BSA and 10 ug/ml palmitic acid and 5 ug/ml cholesterol.
5. BMA with 0.5% (w/v) BSA and 10 ug/ml palmitic acid and 20 ug/ml cholesterol.
6. BMA with 0.5% (w/v) BSA and 10 ug/ml palmitic acid and 100 ug/ml cholesterol.
7. BMA with 0.5% (w/v) BSA and 10 ug/ml palmitic acid and 1% (v/v) Bacto-PPLO serum fraction.
8. BMA with 15% (v/v) inactivated swine serum.

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1 Sigma Chemical Co Ltd., St Louis, MO, U.S.A.
2 Koch-Light Ltd., Haverhill, Suffolk, U.K.

Procedure:

The test strains were grown in MB for 48 hours at 37°C and cells were harvested by centrifugation at 2,000 g for 45 minutes at 4°C. The pellets were washed three times in washing medium, recentrifuged after each washing and then finally suspended in 2 ml of the same medium. The suspensions were filtered by passing through a 450 nm pore diameter membrane filter and the filtrates were dispensed in aliquots and stored at -70°C. A viable count was carried out on a thawed suspension using agar medium 8, with the washing medium as diluent, to determine which dilution gave a heavy growth (10^6 CFU/ml) and which gave a sparse to moderate growth (10^2 CFU/ml). For the test proper a set of plates of media 1-8 was inoculated each with five drops of 20 µl of the selected culture dilutions of the organism using the washing medium as diluent. Plates were incubated in candle jars at 37°C and examined every 2 days under the microscope until it was possible to count the number of colonies. *M.gallisepticum* S6LP and *A.laidawii* PG8 were included as positive (sterol requiring) and negative (non-sterol requiring) controls respectively. This experiment was carried out twice using the Sigma cholesterol (C8378) and once again using the Koch-Light product. Finally a small pilot test was conducted using only medium 6 prepared with the Sigma cell culture-tested cholesterol. For this the untyped strains as well *M.gallisepticum* S6LP, *M.gallinarum* PG16 and *M.anatis* 1340 were tested.

Sterol requirement using broth media

Materials:

Washing solution (0.25 M NaCl, 0.01 M $MgCl_2$)

A 1 litre solution was prepared by dissolving 14.61 g sodium chloride and 2.03 g magnesium chloride in 1,000 ml distilled water. It was sterilized by autoclaving at 15 lb/sq.in for 15 minutes.

Basal medium (BMB)

Part A	
Bacto-PPLO broth (w/o crystal violet)	2.1 g
Distilled water	78.5 ml
Part B	
Yeast extract (25% w/v)	10.0 ml
D-glucose (10% w/v)	5.0 ml
L-arginine (10% w/v)	4.2 ml
Benzyl penicillin (200,000 i u/ml)	0.25 ml

The ingredients of part A were sterilized by autoclaving at 15 lb/sq.in for 15 minutes and, after cooling, were mixed with part B. The basal medium was supplemented with various additives described below, and the proportion of distilled water was adjusted accordingly to maintain the concentration of the ingredients of the medium. The supplements are given below.

Bovine serum albumin fraction V and palmitic acid

Stock solutions were prepared as described for the tests using agar media.

Tween 80

Stock solution of 10% (v/v) Tween 80[1] (polyoxyethylenesorbitan monooleate) was prepared in distilled water and sterilized by autoclaving at 15 lb/sq.in for 15 minutes.

Cholesterol solutions

Stock solution containing 20 mg/ml of cholesterol (Sigma cell culture-tested) in ethanol (95%) was stored at 4°C and warmed before use to dissolve any crystals. Various concentrations of cholesterol solution were prepared with Tween 80 and ethanol, as shown in Table 5.1, and 0.2 ml volumes were added to 100 ml BMB to prepare media with cholesterol concentrations of 0, 1.0, 5.0, 10.0 and 20.0 ug/ml. The basal medium and the cholesterol solutions were gently warmed before use.

The cholesterol test broths

The following 8 media were prepared from the basal medium and supplements:

1. Basal medium (BMB)
2. BMB with 0.5% (w/v) BSA and 10.0 ug/ml palmitic acid.
3. BMB with 0.5% (w/v) BSA, 10 ug/ml palmitic acid and 1.0 mg/ml Tween 80.
4. BMB with 0.5% (w/v) BSA, 10.0 ug/ml palmitic acid, 1.0 mg/ml Tween 80 and 1.0 ug/ml cholesterol.
5. BMB with 0.5% (w/v) BSA, 10.0 ug/ml palmitic acid, 1.0 mg/ml Tween 80 and 5.0 ug/ml cholesterol.
6. BMB with 0.5% (w/v) BSA, 10.0 ug/ml palmitic acid, 1.0 mg/ml Tween 80 and 10.0 ug/ml cholesterol.
7. BMB with 0.5% (w/v) BSA, 10.0 ug/ml palmitic acid, 1.0 mg/ml Tween 80 and 20.0 ug/ml cholesterol.
8. BMB with 1% (w/v) Bacto-PPLO serum fraction.

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[1] Aldrich Chemical Co Ltd., Gillingham, Dorset, U.K.

Table 5:1 Preparation of cholesterol solutions

Cholesterol stock (ml)	+ Tween 80 stock (ml)	+ Ethanol (ml)	= Cholesterol concentration mg/ml
0.0	1.0	1.0	0.0
0.05	1.0	0.95	0.5
0.25	1.0	0.75	2.5
0.5	1.0	0.5	5.0
1.0	1.0	0.0	10.0

Procedure:

Because of the problems encountered with the test using agar media, it was decided to conduct a pilot test by the broth method using *M.gallisepticum* S6LP before a full scale experiment was planned.

A broth culture of the organisms was prepared in medium 8 and a 3% (v/v) inoculum of the culture (at logarithmic phase) was used to inoculate 100 ml of each of the eight test media. They were incubated at 37°C and the growth was observed daily. When turbidity was seen in cultures at the higher cholesterol concentration (3-10 days) the organisms were harvested by centrifugation at 34,000 g for 15 minutes at 4°C and the pellets were washed twice in washing solution. The cell pellets were then frozen at -70°C until the protein assay was carried out. This was performed on pellets from each of the eight media using the Pierce BCA protein assay reagent[1].

5.2.2 Sensitivity to digitonin

The sensitivity to digitonin was used as an indirect indication of sterol requirement. The method was as described by Erno and Stipkovits (1973) using filter paper discs impregnated with digitonin.

Materials:

Digitonin

Stock solution of 1.5% (w/v) digitonin[2] in ethanol was prepared. It was warmed gently in a 56°C water bath for 30 minutes to dissolve the digitonin completely.

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1 Pearce Chemical Co, Rockford, Illinois, U.S.A.

2 BDH Chemicals Ltd, Poole, Dorset, U.K.

Sterile blank filter paper sensitivity discs[1] (6 mm diameter) were impregnated with 20 µl of the digitonin solution, placed in a sterile plastic Petri dish and dried overnight at 37°C. They were stored at 4°C.

Procedure:

Mycoplasma agar plates (7 ml) were dried at 37°C before inoculation to remove the surface moisture. They were then inoculated with 2 dilutions of the test organisms, containing approximately 10^5 and 10^3 CFU/ml, using the running drop technique (Freundt *et al.*, 1979). When the the inoculum had been absorbed a digitonin disc was placed in the centre of the run. The plates were incubated at 37°C in a candle jar and examined for evidence of growth inhibition zones around the discs. The width of the zone was measured from the edge of the disc to the edge of the area where mycoplasma growth occurred.

M.gallisepticum S6LP and *A.laidlawii* PG8 were included as known positive and negative controls.

5.2.3 Sensitivity to sodium polyanethol sulphonate (SPS)

Sensitivity to SPS is used as an indirect indication of sterol requirement in *Mollicutes*. The basis of the reaction is thought to be due to precipitation of cholesterol in the sterol requiring mycoplasmas, thus causing lysis (Erno and Stipkovits, 1973). The method used was that described by Freundt *et al.* (1973)

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1 Difco Laboratories, Detroit, Michigan, U.S.A.

Materials:

Sodium polyanethol sulphonate (SPS)

Stock solutions of 5% and 20% (w/v) SPS[1] in sterile distilled water were prepared. Sterile blank filter paper discs were impregnated with these solutions as described for the digitonin test.

Procedure:

The procedure was as described for the digitonin test.

5.2.4 Urea hydrolysis

The test for hydrolysis of urea was done by two methods. The first was a broth method described by Livingston, (1972) and based on the production of ammonia and carbon dioxide resulting from the hydrolysis of a urea supplement in the medium. The accompanying increase in pH of the culture medium is demonstrated by colour change of the phenol red indicator.

The second method was carried out in agar medium as described originally by Shepard and Howard (1970). The test is based on the formation of manganous dioxide from manganous chloride in the prescence of ammonia which is liberated as a result of the hydrolysis of urea by the urease activity of the organisms. Deposition of the insoluble manganous dioxide on the surface of the colonies gives them a dark brownish colour.

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1 Sigma Chemical Company, St Louis, MO, U.S.A.

Urea hydrolysis test in broth medium

Materials:

Urea broth medium (UBM)

Part A	
Tryptic digest broth[1] (1.6% w/v)	100.0 ml
Sodium chloride (1% w/v)	100.0 ml
Glucose	0.2 g
Part B	
Horse serum (uninactivated)	58.0 ml
Yeast extract (25% w/v)	29.0 ml
Urea (10% w/v)	0.85 ml
Phenol red (1% w/v)	0.3 ml
Benzylpenicillin (100,000 iu./ml)	1.7 ml

Part A was prepared and the pH was adjusted to 6.0. It was autoclaved at 15 lb/sq.in for 15 minutes and allowed to cool. The ingredients of part B were mixed, adjusted to pH 6.0, Seitz-filtered and added aseptically to part A. The medium was stored at 4°C. A control medium (UBC) was prepared as above but without addition of urea.

Procedure:

UBM and UBC were dispensed in sterile vials in 1 ml aliquots. A vial of each medium was inoculated with a colony bearing agar block (MA without glucose or arginine) of a 48 hour culture of the test organisms. A vial of each medium was also inoculated with a sterile agar block (pH 6.0) as a negative control. All vials were

1 Difco Laboratories, Detroit, Michigan, U.S.A.

sealed with Parafilm and incubated at 37°C. They were examined every 12 hours for up to 4 days and any change in the pH was read by colour comparison with a set of standards ranging from 6.0 to 7.8. A positive result was recorded when a difference of more than 0.5 of a pH unit was seen between the UBM of the test culture and the negative control. The test was considered invalid if the inoculated UBC or uninoculated controls exhibited the same colour change as the test culture. Avian *Ureaplasma gallorale* D6-1 and *M.gallisepticum* S6LP were included as known positive and negative controls.

Urea hydrolysis test in agar medium

Materials:

Urea agar medium (UAM)

The preparation of this medium was as for UBM but included 2.26 g Lab M agar powder in part A. After autoclaving and allowing to cool to 56°C, the sterile ingredients of part B, omitting the phenol red, were mixed aseptically and warmed to 56°C. Parts A and B were mixed and dispensed in 8 ml aliquots into sterile 5 cm diameter plastic Petri dishes.

Indicator reagent

A solution containing urea 1% (w/v) and manganous chloride[1] ($MnCl_2$. $4H_2O$) was prepared in sterile distilled water.

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1 BDH Chemicals Ltd., Poole, Dorset, U.K.

Procedure:

Strains 4229 and B2/85, together with *U.gallorale* D6-1 and *M.gallisepticum* S6LP as known positive and negative controls, were adapted to UAM by several passages. Then fresh UAM plates were inoculated in triplicate using colony-bearing agar blocks and incubated in anaerobic conditions in Gas Pak jars. When colonies were seen, one or two drops of the indicator were added at room temperature to the surface of one of the agar plates of each test organism and the reactions read immediately under the microscope. Colonies which exhibited a dark brownish colour within 5-10 seconds were considered positive while unstained colonies were negative. The test was repeated at 48 hours and 5 days incubation using the second and third agar plates.

5.2.5 Arginine hydrolysis

The method for detection of arginine hydrolysis was as described by Aluotto *et al.* (1970) based on the demonstration of the presence of a three enzyme dihydrolase pathway which includes arginine deiminase, ornithine carbamoyltransferase (ornithine transcarbamylase) and carbamoyl phosphokinase. The arginine substrate in the medium is first converted to citrulline then into ornithine and carbamoyl phosphate. The latter is hydrolysed and results in release of ammonia, carbon dioxide and high energy phosphate (ATP) (Schimke and Barile, 1963). The subsequent increase in pH of the medium is indicated by colour change of the phenol red indicator.

Materials:

Arginine broth medium (ABM)

This medium was prepared as serum fraction broth (2.3) except that the glucose was omitted and the pH was adjusted to 7.0. A control medium (ABC) was also prepared as above but without addition of glucose or arginine.

Procedure:

The method of testing the untyped mycoplasma strains for arginine hydrolysis was as described in hydrolysis of urea in broth medium (5.2.4). *M.gallinarum* PG16 and *M.gallisepticum* S6LP were included as known positive and negative controls respectively. A positive result was indicated by an increase in pH of more than 0.5 unit in cultures in ABM in comparison with cultures in ABC.

5.2.6 Glucose breakdown

Two methods were used for determining glucose breakdown. The first was the indirect method described by Aluotto *et al.* (1970) which is based on the demonstration of a fall in pH due to acid production during the growth of organisms in medium containing glucose.

The second method was the direct test described by Edward and Moore (1975) which determines disappearance of glucose from the medium. To demonstrate this, the amount of glucose remaining in the growth medium is assayed at various time intervals by the glucose oxidase method. In this assay the glucose is converted to gluconic acid and hydrogen peroxide in the presence of the enzyme glucose oxidase. The hydrogen peroxide can oxidise a suitable oxygen acceptor to produce chromogenic products in the presence of peroxidase enzyme. The intensity of colour

of these products is proportional to the amount of glucose in the medium (Hugget and Nixon, 1957; Trinder,1969).

Indirect test for glucose breakdown

Materials:

Glucose broth medium (GBM)

This medium was prepared as serum fraction broth (2.3) except that arginine was omitted and the pH was adjusted to 7.8. A control broth medium (GBC) was prepared as above, but without addition of glucose or arginine, and the pH adjusted to 7.8.

Procedure:

The method was as described for hydrolysis of urea in broth medium (5.2.4). *M.gallisepticum* S6LP and *M.gallinarum* PG16 were included as known positive and negative controls respectively. A positive result consisted of a fall in pH of 0.5 units or more in cultures containing glucose in comparison with the negative GBC control.

Direct test for glucose breakdown

Materials:

Glucose test broth I

Part A

Bacto-PPLO broth powder	1.47 g
Distilled water	71.0 ml

Part B

Horse serum (unheated)	15.0 ml
Yeast extract (25% w/v)	10.0 ml
Glucose (20% w/v)	0.5 ml
Thallium acetate (5% w/v)	1.0 ml
Penicillin (200,000 iu/ml)	0.5 ml
Phenol red (0.1% w/v)	2.0 ml

The ingredients of part A were mixed, sterilized by autoclaving at 15 lb/sq.in for 15 minutes, allowed to cool below 56^{0}C then mixed aseptically with the sterile ingredients of part B.

Glucose test broth II

This medium was prepared as glucose broth I but without addition of phenol red, and the distilled water was increased to 73 ml to maintain the same concentration of other ingredients.

Glucose test broth III

Part A

Bacto-PPLO broth powder	1.47 g
Distilled water	52.25 ml

Part B

Horse serum (unheated)	15.0 ml
Yeast extract (25% w/v)	10.0 ml
Glucose (20% w/v)	1.25 ml
Thallium acetate (5% w/v)	1.0 ml
Penicillin (200,000 iu /ml)	0.5 ml
Tris buffer (0.5 M)	10.0 ml
Magnesium chloride (0.1 M)	10.0 ml

The ingredients of part A were mixed and sterilized by autoclaving at 15 lb/sq.in for 15 minutes, allowed to cool below 56°C then mixed aseptically with the ingredients of part B.

Tris buffer

Solution A was prepared by dissolving 242 g Tris (hydroxymethyl) aminomethane[1] in 1 litre of distilled water. Solution B was 2M hydrochloric acid.

The pH of 50 ml of solution A was adjusted to 7.8 using solution B and then made up to 200 ml with distilled water. It was autoclaved at 15 lb/sq.in for 15 minutes.

Reagents for glucose oxidase test

These consisted of a commercially-produced kit[2] using the GOD-PAP method (Trinder, 1969).

Deproteinising solution: uranyl acetate (URAC) 0.16% solution.
Reagent mixture: "buffer/enzymes/4-aminophenazone plus phenol".
Standard glucose preparation: "Precimat® Glucose" containing 100 mg/100 ml.

Procedure:

The test organisms were passaged daily 8 times in glucose test broth I using 10% (v/v) inoculum at each passage. The culture (150 ml) was then used to inoculate 1.5 litres of glucose test broth II. It was incubated at 37°C for 1-2 days or until turbidity was evident. The cultures were harvested by centrifugation at 2,000 g for 45 minutes, and the supernatant was decanted aseptically. The cell pellets were

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1 Aldrich Chemical Company Ltd., Dorset, U.K.
2 Boehringer Mannheim GmbH Diagnostica, Germany.

resuspended in a small volume of glucose test broth II (approximately 6 ml) to provide a dense suspension. A small portion of this suspension was removed for estimation of the number of CFU/ml (2.8). A minimum of 10^9 CFU/ml is required (Edward and Moore, 1975). The remaining volume of the suspension (5 ml) was used to inoculate 20 ml of the glucose test broth III and incubated at 37°C together with uninoculated control broth III. After inoculation, duplicate 3 ml samples were removed from both, immediately and at intervals up to 7 days of incubation, for glucose determination by the glucose-oxidase method. *M.gallisepticum* S6LP and *M.gallinarum* PG16 were included in the test as known positive and negative controls respectively.

For each sampling, 0.1 ml aliquots were taken from inoculated and control samples and mixed with 1.0 ml deproteinizing solution (URAC). After mixing by flushing with a pipette several times, the mixture was centrifuged and the clear supernatant removed and used for the assay. For the assay, 0.2 ml of supernatant was mixed with 2.0 ml of the reagent mixture. A glucose standard was prepared by mixing 0.2 ml of the standard glucose solution with 2.0 ml of the reagent mixture. A reagent blank was prepared by mixing 0.2 ml distilled water with 2.0 ml of the reagent mixture.

All samples were then incubated at 20-25°C for 35-60 minutes and the absorbance of the sample and standard against the blank were measured using a Spectrophotometer [1] set at a wavelength of 546 nm. The glucose concentrations were determined by comparison of their absorbance with that of the standard glucose.

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1 Shimadzu Corporation, Kyoto, Japan

5.2.7 Phosphatase activity

The test for phosphatase activity is an optional one for providing description of a new species belonging to the class *Mollicutes* (Subcommittee on the Taxonomy of class Mollicutes, 1979).

Three methods have been described. The first was described by Aluotto *et al.* (1970) and is one in which the substrate sodium phenolphthalein diphosphate is incorporated in the agar medium. In the presence of phosphatase enzyme, the substrate is hydrolysed into phenolphthalein and is subsequently detected by its reaction with added sodium hydroxide, giving a pink-red colour. The second method uses the same substrate but is performed in broth culture (Burger *et al.*,1967; Freundt *et al.*,1979). The third method uses a fluorogenic substrate (4 methylumbelliferyl-phosphate) which is hydrolysed by phosphatase to produce the fluorescent compound 4-methylumbelliferone (Bradbury, 1977). This can be detected by ultra violet radiation. The first of these methods was used here.

Phosphatase activity on agar medium

Materials:

Phosphatase agar medium (PAM)

Part A

Heart infusion broth[1]	1.8 g
Lab M agar No. 2 powder	1.0 g
Distilled water	74.0 ml

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1 Difco Laboratories, Detroit, Michigan, U.S.A.

Part B

Horse serum	20.0 ml
Yeast extract (25% w/v)	5.0 ml
Thallium acetate (5% w/v)	1.0 ml
Sodium phenolphthalein diphosphate[1] (1% w/v)	1.0 ml
Benzyl penicillin (200,000 iu/ml)	0.5 ml

The ingredients of part A were sterilized by autoclaving at 15 lb/sq.in for 15 minutes and allowed to cool to 56°C. The serum and yeast extract were heated at 60°C for one hour before addition to the medium in order to inactivate any phosphatase enzyme in them. Then the ingredients of part B were mixed aseptically and warmed to 56°C, added to part A and dispensed into sterile disposable Petri dishes in 8 ml aliquots.

Procedure:

Triplicate agar plates were inoculated with a 0.02 ml drop of a 24 hour broth culture of each of the untyped strains. *M.meleagridis* 17529 and *M.gallisepticum* S6LP were included as known positive and negative controls, together with an uninoculated plate as a control for the test medium. Plates were incubated in candle jars at 37°C. At 3, 7, and 14 days one of each set of plates was tested by flooding the agar surface with 5 M sodium hydroxide. The appearance of a pink-red colour within 30 seconds in and around the colonies indicated a positive reaction.

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1 BDH Chemicals Ltd., Poole, Dorset, U.K.

5.2.8 Liquefaction of coagulated serum

The test for proteolytic activity is another of the optional tests recommended for characterization of new species of the class *Mollicutes* (Subcommittee on the Taxonomy of class *Mollicutes*, 1979) although only a few *Mycoplasma* species have so far been found to possess this property. Proteolytic ctivity can be demonstrated by liquefaction of gelatin, hydrolysis of casein or by liquefaction of coagulated serum (Aluotto et al.,1970; Freundt, 1983c). Liquefaction of coagulated serum was the method used here.

Materials:

Serum digestion medium (SDM)

Part A	
Heart infusion broth	0.25 g
Distilled water	10.0 ml
Part B	
Horse serum	30.0 ml
Yeast extract (25% w/v)	0.8 ml
Distilled water	1.2 ml

The ingredients of Part A were sterilized by autoclaving at 15 lb/sq.in for 15 minutes and then cooled to below 56^{o}C. The sterile ingredients of Part B were added to 8 ml of Part A. The pH was adjusted to 7.8 with 1 M sodium hydroxide and the medium was dispensed in 2 ml volumes in screw capped tubes. The tubes were placed, with their caps loosened, in a slanted position and heated in flowing steam for 45 minutes until the medium had solidified. They were transferred to a hot air oven at 60^{o}C to dry overnight. The caps were then tightened and the tubes stored at 4^{o}C.

Procedure:

Stock frozen cultures of strains 4229 and B2/85 were used to inoculate slopes of SDM. For each culture one slope was inoculated with undiluted stock containing approximately 10^6 CFU/ml and another with a dilution containing 10^3 CFU/ml. *M.capricolum* California kid and *M.gallisepticum* S6LP were included as positive and negative controls respectively. Two tubes of SDM were inoculated with sterile MB as negative controls. All tubes were incubated at 37°C and read daily for 2 weeks. Liquefaction was shown by the development of a shallow depressed area with a moist base, when the growth was confluent, and by small pits when the growth was more scattered. Accumulation of fluid in the angle between the base of the slope and the wall of the tube can be seen if there is extensive liquefaction.

5.2.9 Production of films and spots

The production of films and spots has been demonstrated by some species of *Mycoplasma* during their growth in solid medium. The films consist of cholesterol and phospholipids while the spots are deposits of calcium and magnesium salts of fatty acids which are liberated by lipolytic activity of the mycoplasmas. In order to detect these reactions, the organisms are best grown in medium containing egg yolk (Fabricant and Freundt, 1967).

Materials:

Egg yolk agar medium (EYAM)

Part A

Bacto-PPLO (w/o crystal violet)	1.47 g
Lab M agar No 2	1.0 g
Distilled water	70.0 ml

Part B	
Swine serum	5.0 ml
Yeast extract (25% w/v)	10.0 ml
Egg yolk emulsion[1]	10.0 ml
NAD (1% w/v)	1.0 ml
Glucose (10% w/v)	1.0 ml
Thallium acetate (5% w/v)	1.0 ml
Benzyl penicillin (200,000 iu /ml)	0.5 ml

Part A was sterilized by autoclaving at 15 lb/sq.in for 15 minutes then allowed to cool to 56°C. The ingredients of part B were mixed aseptically, warmed to 56°C then added to part A. The medium was dispensed into Petri dishes in 8 ml aliquots.

Procedure:

Between 20 and 30 µl of the test organisms were inoculated onto EYAM using a 24 hour broth culture. *M.gallinarum* PG16 and *M.gallisepticum* S6LP were included in the test as known positive and negative controls respectively. An uninoculated agar plate was also included as a medium control. The plates were incubated at 37°C in a candle jar for two weeks and examined daily for presence of the films and spots on the agar surface.

5.2.10. Tetrazolium reduction

Reduction of triphenyltetrazolium chloride into its insoluble red coloured formazan has been demonstrated by some *Mycoplasma* species and is indicative of the

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1 Oxoid Limited, Basingstoke, Hampshire, U.K.

presence of oxidative enzyme activity in these organisms causing the reduction of the substrate. The method used was described by Aluotto *et al.* (1970) utilizing agar medium containing the tetrazolium chloride substrate.

Materials:

Tetrazolium agar medium (TAM)

Part A	
Heart infusion agar	4.0 g
Distilled water	74.0 ml
Part B	
2,3,5 triphenyltetrazolium chloride (2% w/v)	1.0 ml
Horse serum (inactivated)	20.0 ml
Yeast extract (25% w/v)	5.0 ml

Part A was sterilized by autoclaving at 15 lb/sq.in for 15 minutes and allowed to cool to 56°C. The ingredients of part B were mixed aseptically, warmed to 56°C and added to part A. The medium was dispensed into Petri dishes in 8 ml aliquots.

Reagent

A 2% (w/v) aqueous solution of 2, 3, 5 triphenyltetrazolium chloride[1] was prepared and sterilized by Seitz filtration.

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1 BDH Chemicals Ltd., Poole, Dorset, U.K.

Procedure:

Stock frozen cultures of strains 4229 and B2/85 were used to inoculate MA. Undiluted thawed stock, which contained approximately 10^6 CFU/ml, and a 10^{-3} dilution of this were used as inocula. After colonies had grown (3-4 days), colony-bearing blocks from each plate were used to inoculate duplicate TAM plates. One plate was incubated aerobically, and the other anaerobically for 2 weeks and examined every 2 days for the development of a pink to dark red colour. *M.gallisepticum* S6LP and *M.iners* PG30, incubated both aerobically and anaerobically, were included as positive and negative controls.

5.2.11 Hydrolysis of aesculin and arbutin

Hydrolysis of aesculin and arbutin by the enzyme ß-D-glucosidase has been detected in some species of *Acholeplasma* and was considered a useful test to differentiate them from the *Mycoplasma* species (William and Wittler, 1971; Rose and Tully, 1983). In the presence of ß-D-glucosidase, aesculin (i.e ß-D-glucoside) is hydrolysed into glucose and 6,7-dihydroxycoumarin, the latter product develops a brownish-black compound on combination with ferric ions (ferric citrate) incorporated into the medium. Arbutin (i.e. ß-D-glucopyranoside) is hydrolysed to glucose and hydroquinone and the latter product develops a brownish-black compound with ferric ions.

The test used was that developed by Askaa (Freundt *et al.* 1979) in which paper discs are impregnated with the substrate and ferric citrate. This has the advantage that conventional medium can be used without the need to incorporate the test substrate and the reagent as in the method described by Williams and Wittler (1971).

Materials:

An aqueous solution of ferric citrate (5% w/v) was prepared and 20 µl used to impregnate sterile blank filter paper discs (6 mm in diameter). The discs were dried overnight at 37°C in a sterile Petri dish and then further impregnated with 20 µl of an aqueous solution (10% w/v) of either aesculin [1] or arbutin[2]. They were dried as before and stored at 4°C.

Procedure:

MA plates were inoculated with two dilutions of the test organisms as described for the tetrazolium test using the running drop method. Following absorption of the inoculum, impregnated discs of either aesculin or arbutin were placed in the centre of the inoculated run, and the plates incubated at 37°C for 7 days. The development of a brownish-black colour in the culture streak around the disc indicated a positive reaction. *A.axanthum* S-743 and *M.gallisepticum* S6LP were included as positive and negative controls respectively.

5.2.12 Haemadsorption and haemagglutination

The interaction between mycoplasmas and erythrocytes is well recognised. In haemadsorption, the erythrocytes adsorb to mycoplasma colonies whereas in haemagglutination the mycoplasmas in suspension adhere to and agglutinate the erythrocytes. The test for adsorption of erythrocytes, preferably using cells from various animal species, is one of the optional tests recommended by the Subcommittee on the Taxonomy of the class *Mollicutes* (1979) to obtain additional information for the description of a new species.

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1 BDH Chemicals Ltd., Poole, Dorset, U.K.

2 Sigma Chemical Company, St Louis, MO, U.S.A.

Haemadsorption test (HAd)

The method for detecting attachment of erythrocytes to the mycoplasma colonies was based on that outlined by Gardella and DelGiudice (1983).

Materials:

Erythrocytes

Chicken, turkey and duck erythrocytes were collected from the wing vein into 10% (v/v) citrate saline (2.4). They were washed three times in PBS, pH 7.0, prepared as a 0.5% (v/v) suspension of the packed cells and stored at 4°C for up to 7 days.

Procedure:

Serial tenfold dilutions of the culture under test were prepared in MB from frozen stocks to provide a minimum of 50 well-separated colonies when inoculated onto MA. After inoculation the MA plates were incubated at 37°C for 3 to 4 days depending on the growth rate of the organism. When the colonies were of medium size (100 - 150 µm diameter) 2 ml of the erythrocyte suspension was poured carefully onto the agar surface. The plates were then incubated at 37°C for 30 minutes. The erthyrocyte suspension was gently poured off and the agar surface carefully washed with 5-10 ml of PBS. The colonies were observed for haemadsorption at X100 magnification. This procedure was carried out using erthrocytes from chickens, turkeys and ducks and *M.gallisepticum* S6LP was included as a positive control.

Haemagglutination test (HA)

The method used was as described by Gardella and DelGiudice (1983) with slight modification.

Materials

Erythrocytes

A 0.75% (v/v) suspension of chicken erythrocytes in PBS was prepared as described above.

Mycoplasma suspension

The test organisms were grown in broth for 48 hours. The broth consisted of MB except that the swine serum and yeast extract supplements were reduced to 5%. The organisms were harvested by centrifuging at 3,000 g for 45 minutes and were suspended in PBS to one hundreth of the original culture volume.

Procedure:

Serial twofold dilutions of the test strains were made in 25 µl volumes in 96 U-well plastic plates[1] using a digital multichannel pipette[2], then 25 µl of erythrocyte suspension was added to each well, followed by 25 µl of PBS. *M.gallisepticum* S6LP was also included. The plate was agitated for 10 seconds on a mixer[3] and then incubated at room temperature for 30 minutes. Haemagglutination reactions were recorded, 1 HA unit being the highest dilution that gave 100% agglutination.

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1 Sterilin, Hounslow, Middlesex, U.K.

2 Titertek, Flow Laboratories, Finland

3 TW Suspension Mixer, Luckham, Sussex, U.K.

5.2.13 Determination of the DNA base composition

Determination of the guanine plus cytosine (G +C) content of the DNA of the organisms is proposed as a minimal requirement for characterization of new species of the class *Mollicutes* (Subcommittee on Taxonomy of class *Mollicutes*, 1979).

Facilities for determining this property were not available in this laboratory but the DNA base composition of strain 4229 had already been determined by Dupiellet *et al.* (1990) using both the melting temperature and buoyant density methods.

5.3 Results

5.3.1 Sterol requirement

Sterol requirement using agar media

The results of the test for sterol requirement using the Sigma C8373 reagent are given in Table 5:2. Neither of the untyped strains nor the sterol requiring positive control *M.gallisepticum* showed a growth response to cholesterol. Strains 4229, B2/85 and *M.gallisepticum* all grew well in basal medium supplemented with bovine albumin, palmitic acid and 1% PPLO serum fraction or in basal medium with 15% swine serum. In contrast, *A.laidlawii* grew in all the media irrespective of the cholesterol concentration. Two repeats of this test, including one using a different source of cholesterol (Koch-Light), yielded similar results.

In a subsequent pilot test using another cholesterol preparation (Sigma cell culture-tested), strains 4229 and B2/85 did demonstrate very slight growth on medium containing 100 µg/ml cholesterol but there was no growth of the positive control organisms (*M.gallisepticum, M.gallinarum* and *M.anatis*) that were included. For this

Table 5:2 Sterol requirement using agar media

Organism	Inoculum CFU/ml	1 Basal medium	2	3	4	5	6	7	8 Basal medium with swine serum
			Basal medium with 0.5%(w/v) BSA and 10 µg/ml palmitic acid						
			Cholesterol conc. (ug/ml)					1% PPLO serum fraction	
			0	0.2	5	20	100		
4229	10^6	-[1]	-	-	-	-	-	2+	3+
	10^2	-	-	-	-	-	-	1+	2+
B2/85	10^6	-	-	-	-	-	-	2+	3+
	10^2		-	-	-	-	-	1+	2+
A.laidlawii PG8	10^6	3+	3+	3+	3+	3+	3+	3+	3+
	10^2	1+	1+	1+	1+	1+	1+	1+	2+
M.gallisepticum S6LP	10^6	-	-	-	-	-	-	2+	3+
	10^2	-	-	-	-	-	-	1+	2+

1. Colony numbers were scored as follows: 3+ = profuse, 2+ = moderate, 1+ = sparse, - = no growth

reason the test was not carried out in full and a pilot test by the broth method was conducted.

Sterol requirement using broth media

The results of this pilot test using *M.gallisepticum* are shown in Table 5:3. Growth was not detected in the basal medium with or without bovine albumin, palmitic acid and Tween 80. However, a very limited growth response was apparent in the media with 10 and 20 ug/ml of cholesterol and the cell protein yield was higher at 10 than 20 ug/ml. The organism also showed only limited growth in medium with 1% (v/v) PPLO serum fraction. Since a minimum yield of 1 mg cell protein/100 ml has been proposed before this test is valid (Razin and Tully, 1970) it was not considered worthwhile to proceed with a full scale experiment at this stage.

5.3.2 Sensitivity to digitonin

The untyped strains were both sensitive to digitonin with inhibition zones of between 7 and 8 mm (Table 5:4). *A.laidlawii* exhibited no sensitivity whereas *M.gallisepticum* showed an inhibition zone of 10 mm.

5.3.3 Sensitivity to sodium polyanethol sulphonate

Strains 4229 and B2/85 were sensitive to SPS with inhibition zones of 5 mm and 6 mm with 5% (w/v) and 20% (w/v) respectively (Table 5:4). *A.laidlawii* was resistant to SPS while *M.gallisepticum* was inhibited.

Table 5:3 Sterol requirement using broth media: pilot test using *M.gallisepticum* S6LP

1	2	3	4	5	6	7	8
Basal medium	Basal medium with 0.5%(w/v) bovine albumin and 10 ug/ml palmitic acid						Basal medium with 1% (w/v) serum fraction
	No additives	1mg/ml Tween 80	Tween 80 +1ug/ml cholest.[1]	Tween 80 +5 ug/m cholest.	Tween 80 +10 ug/m cholest.	Tween 80 +20 ug/ml cholest.	
0[2]	0	0	0	0	1.49	0.516	0.858

1. Cholesterol
2. Cell protein yield (mg/100ml)

Table 5:4 The sensitivity to digitonin and sodium polyanethol sulphonate

	Growth inhibition zone (mm)		
Organism	Digitonin 1.5% (w/v)	Sodium polyanethol sulphonate 5% (w/v)	20% (w/v)
4229	7	5	6
B2/85	8	5	6
A.laidlawii	0	0	0
M.gallisepticum	10	4	5

5.3.4 Urea hydrolysis

There was no hydrolysis of urea by the untyped strains or *M.gallisepticum* in either test (Table 5:5). *U.gallorale* demonstrated a positive reaction in both broth and agar after 24 and 48 hours but a weak positive or no reaction after longer incubation.

5.3.5 Hydrolysis of arginine

The untyped strains together with *M.gallisepticum* were all unable to hydrolyse arginine and did not give an alkaline shift in the test medium (Table 5:6). *M.gallinarum* as the positive control organism gave an alkaline shift in the arginine test medium.

5.3.6 Glucose breakdown

Indirect test for glucose breakdown

Both the untyped strains showed ability to break down glucose (Table 5:7). Strain B2/85 appeared to break it down more rapidly since the pH of the medium had fallen to 6.9 by 12 hours. Strain 4229 had reduced the pH to 7.1 by this time. The *M.gallisepticum* positive control culture also produced an acid shift in the test medium while the *M.gallinarum* negative control culture did not.

Direct test for glucose breakdown

The results of the determination of glucose disappearance from the growth medium are given in Figures 5:1-5:4. The inocula of 4229, B2/85, *M.gallisepticum* and *M.gallinarum* contained 1.4×10^9, 2.8×10^9, 2.7×10^9 and 1.3×10^{10} viable organisms respectively.

Table 5:5 Hydrolysis of urea

	Urease activity			
	In broth		In agar medium	
Organisms	24hrs	4 days	48 hrs	5 days
4229	-[1]	-	-	-
B2/85	-	-	-	-
M.gallisepticum	-	-	-	-
Ureaplasma gallorale D6-1	+[2]	+	+	(+)[3]

1. Negative reaction
2. Positive reaction
3. Weak positive reaction

Table 5:6 **Hydrolysis of arginine**

Organisms	Hydrolysis
4229	-
B2/85	-
M.gallisepticum	-
M.gallinarum	+

Table 5:7 **Indirect glucose breakdown**

Organisms	Glucose breakdown
4229	+
B2/85	+
M.gallisepticum	+
M.gallinarum	-

Fig. 5:1 Direct test for glucose breakdown by strain 4229

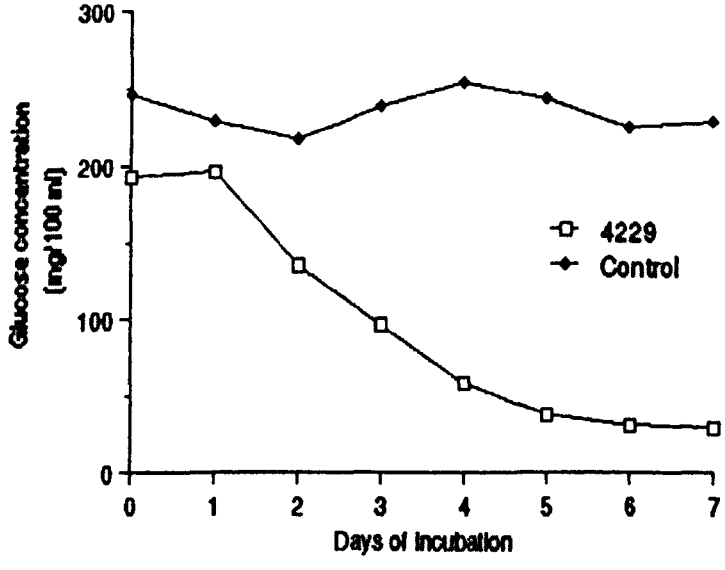


Fig. 5:2 Direct test for glucose breakdown by strain B2/85

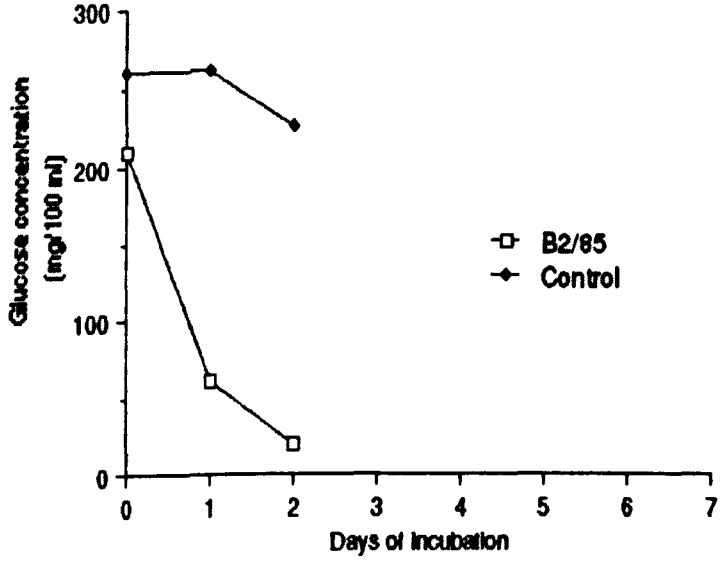


Fig. 5:3 Direct test for glucose breakdown by *M.gallisepticum* S6LP

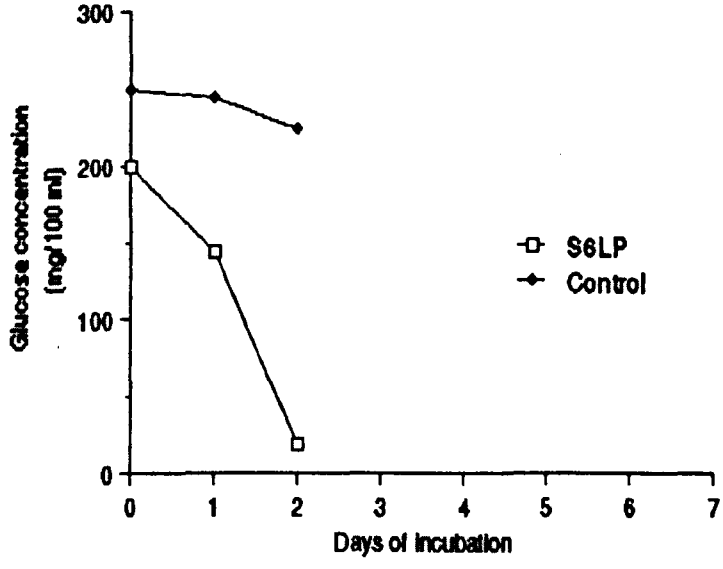
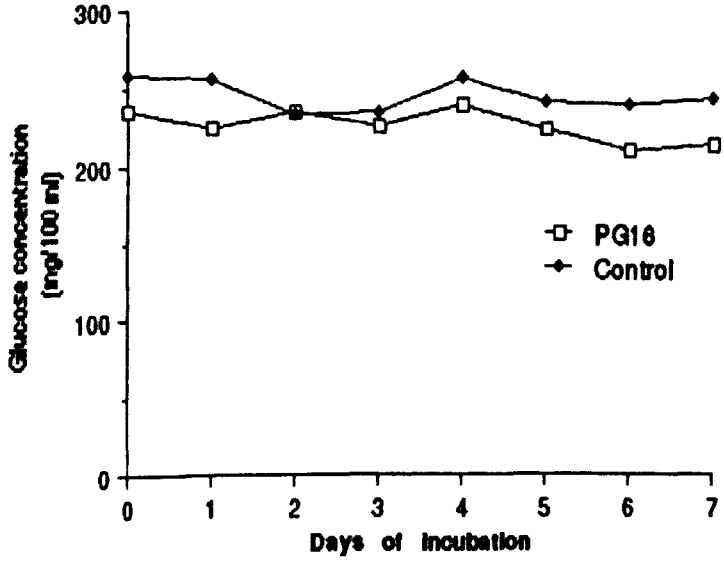


Fig. 5:4 Direct test for glucose breakdown by *M.gallinarum* PG16



Strains 4229 and B2/85 both depleted glucose from the medium but it was more rapid with B2/85 than with 4229. *M.gallisepticum* also exhibited breakdown of glucose from the medium while there was no evidence of depletion by *M.gallinarum*.

5.3.7 Phosphatase activity

The untyped strains showed a weak reaction, but only after 14 days of incubation. The reaction was characterised by a slight pink coloration of the medium which was also seen in the uninoculated control plate after 14 days (Table 5:8). The positive control culture of *M.meleagridis* gave dark red coloration even after 3 days of incubation while the negative control, *M.gallisepticum*, showed no coloration at any time.

5.3.8 Liquefaction of coagulated serum

All the tests, including the uninoculated control, accumulated moisture but the *M.capricolum* positive control culture was the only one to demonstrate moist depressions and pits in the medium (Table 5:9).

5.3.9 Production of films and spots

Neither the untyped strains nor the *M.meleagridis* negative control produced films and spots within the 12 day incubation period. The positive control, *M.gallinarum*, developed a films and spots reaction after 2 days and the intensity increased with duration of incubation (Table 5:10).

Table 5:8 Phosphatase activity

Organisms	Incubation time (days)		
	3	7	14
4229	-[1]	-	(+)[2]
B2/85	-	-	(+)
M.meleagridis	+[3]	+	+
M.gallisepticum	-	-	-
Uninoculated plate	-	-	(+)

1. No colour
2. Slight pink colour
3. Dark red colour

Table 5:9 Liquefaction of coagulated serum

	Evidence of liquefaction			
	Accumulated moisture		Pits/depressions in the medium	
	CFU/ml in inoculum			
Organisms	10^6	10^3	10^6	10^3
4229	+	+	-	-
B2/85	+	+	-	-
M.capricolum	+	+	+	+
M.gallisepticum	+	+	-	-
Uninoculated plate	+	+	-	-

Table 5:10 Production of film and spots

Organisms	Evidence of film and spots Incubation time (days) 1	2	4	6	8	10	12
4229	-	-	-	-	-	-	-
B2/85	-	-	-	-	-	-	-
M.gallinarum	-	2+[1]	2+	3+	4+	4+	4+
M.meleagridis	-	-	-	-	-	-	-
Uninoculated plate	-	-	-	-	-	-	-

1. 2+ to 4+ represents increasing intensityof film and spots reaction

5.3.10 Tetrazolium reduction

Strains 4229 and B2/85 gave a moderately positive reaction under aerobic incubation and a stronger reaction under anaerobic conditions (Table 5:11). The positive control, *M.gallisepticum* also gave moderate or strong reactions while *M.iners* and the uninoculated control plates showed no reactions.

5.3.11 Hydrolysis of aesculin and arbutin

The untyped strains and the negative control, *M.gallisepticum* showed no evidence of hydrolysis of either aesculin or arbutin (Table 5:12). *A.axanthum* as the positive control organism was able to hydrolyse both these substrates.

5.3.12 Haemadsorption and haemagglutination

The untyped strains showed strong haemadsorption with 75 to 100% of the individual colony surface being covered with erythrocytes. The reaction was the same with chicken, turkey and duck erythryocytes and a similar reaction was observed with the positive control organism, *M.gallisepticum*.

In the haemagglutination test, the titer for strains 4229, B2/85 and S6LP was 32, 16 and 32 respectively.

5.3.13 Determination of the DNA base composition

The G + C content of the DNA of strain 4229 measured by Dupiellet *et al.* (1990) was 31.9 mol % by both buoyant density and by melting temperature.

Table 5:11 **Tetrazolium Reduction**

	Aerobic incubation		Anaerobic incubation	
	CFU/ml in inoculum			
Organisms	10^6	10^3	10^6	10^3
4229	2+[1]	2+	3+[2]	3+
B2/85	2+	2+	3+	3+
M.gallisepticum	3+	2+	3+	3+
M.iners	-	-	-	-
Uninoculated control	-	-	-	-

1. Moderate positive reaction
2. Strong positive reaction

Table 5:12 ß-D-glucosidase activity

	Aesculin hydrolysis		Arbutin hydrolysis	
	CFU/ml in inoculum			
Organisms	10^6	10^3	10^6	10^3
4229	-	-	-	-
B2/85	-	-	-	-
A.axanthum	3+[1]	2+	2+	1+
M.gallisepticum	-	-	-	-

1. 1+ to 3+ represents increasing intensity of brown colour

5.4 Discussion

The test for sterol requirement of mycoplasmas for growth is necessary for their characterization (Subcommittee on the Taxonomy of *Mollicutes*, 1979). The requirement for sterol was originally regarded as one of the important characteristics that differentiated the *Mycoplasma* species from bacteria (Edward and Fitzgerald, 1951) and their L-phase variants (Razin and Tully, 1970) which do not require sterol for growth. Sterol requirement was considered one of the reasons to separate the order *Mycoplasmatales* from the *Schizomycetes* and to establish them as a new class called *Mollicutes* (Edward and Freundt, 1967). In addition, the sterol requirement distinguishes the families *Mycoplasmataceae* and *Spiroplasmataceae* from the *Acholeplasmataceae* (Razin and Tully, 1970; Edward, 1971; Subcommittee on the Taxonomy of *Mollicutes*, 1979).

An attempt was made to assess the sterol requirement of strains 4229 and B2/85 by showing a quantitative growth response to increasing levels of cholesterol. The test was attempted first using agar media but unfortunately it was unsuccessful. There was no growth of these strains, or of the sterol requiring species used as a control, except in the media containing serum fraction or whole serum. The use of a cholesterol preparation from a different source did not influence the results. A subsequent pilot test using a third different cholesterol preparation did show very limited growth of the untyped organisms on agar medium containing 100 µg /ml cholesterol but no growth was obtained with three known sterol requiring species even though Razin and Tully (1970) have shown that these three species (*M.gallisepticum, M.gallinarum* and *M.anatis*) grow well at lower cholesterol concentrations.

Several possible reasons might have caused the lack of growth response of the organisms to cholesterol in agar media. For example, there might have been some

inhibitory factor or factors in the medium ingredients, but the results indicated that neither the basal medium, nor the mixture of basal medium and bovine albumin and palmitic acid, had an inhibitory effect on growth. Edward (1971) found toxic material, possibly fatty acid, in different batches of Difco-PPLO agar base for preparing the basal medium for this test. This is not considered a likely factor to explain our results because we used Difco-PPLO broth base with addition of an agar that we know to support good mycoplasma growth. Moreover Edward found that growth of *A.laidlawii* was inhibited in his basal medium and we did not.

Another possible reason for the failure of the organisms to grow in the presence of cholesterol is related to technical difficulties in the preparation of the cholesterol stock solution as several attempts are sometimes necessary to prepare a finely dispersed aqueous suspension (Edward and Fitzgerald, 1951). In our study care was taken to use only suspensions appearing to be homogeneous.

Also with regard to the stock solution of cholesterol, it is possible that the type of cholesterol might influence the results. However, as mentioned above, three different cholesterol reagents were investigated and none of them gave satisfactory results. Therefore it is unlikely that the type of cholesterol used accounted for the lack of growth response.

In a recent report by Hill (1991) describing a new species of mycoplasma, *M.oxoniensis*, a modification of the agar plate test was used to demonstrate a growth response to cholesterol. The plates were seeded with a single large colony and then similar subculture was made on the appropriate medium for three passages to ensure that the growth was not due to a carryover of serum from the original growth medium. It will be useful to try this method with our strains.

In the pilot test using broth medium *M.gallisepticum* showed a very limited yield of cell protein in the two media with the highest concentrations of cholesterol (10 ug/ml and 20 µg/ml) moreover the yield in the latter medium and in the broth containing serum fraction were both lower than that from the broth containing 10 µg/ml cholesterol. These results do not compare with those of earlier workers who, using this method and the same strain *M.gallisepticum*, obtained considerably higher protein yields (Razin and Tully, 1970; Forrest, 1982).

There are a number of reasons that might account for these unexpected results. For example there may have been some loss of cell protein during the centrifugation and washing process. Another possible problem may be the use of Tween 80. It has been found that the growth of several mycoplasma species can be inhibited by this detergent by the lytic effect to these wall-less organisms (Razin and Tully, 1970). However the apparent lack of growth of the organisms in the medium without Tween 80 (basal medium with serum fraction) suggests that this is not a likely explanation for the results. This detergent could be omitted and the cholesterol dissolved in ethanol only, but unfortunately time did not permit further experiments.

Another explanation for the low cell protein yield in our experiment could be the slow growth of the organisms and the use of a low volume of inoculum (3% v/v). The use of a larger inoculum has been suggested by Razin and Tully (1970) if yields are unsatisfactory. These workers also suggest an increase in the bovine serum albumin content of the media from 0.5 to 1% in order to increase yield.

In summary, the recommended tests for sterol utilization of strains 4229 and B2/85 were not satisfactorily completed. In one pilot test by the agar method they were capable of very limited growth, but further investigation is needed to confirm this result and to resolve the reasons for the failure of these methods.

Other workers may well have encountered similar problems. For example Erickson *et al.* (1986), in their characterization of *M.hyopharyngis* as a new *Mycoplasma* species from swine, used the agar plate method of Edward (1971) for evaluation of sterol requirement and found that four out of seven strains, including the type strain, did not grow on medium supplemented with 1% PPLO serum fraction or various amounts of cholesterol, although they grew in the presence of 20% horse serum. All seven strains were inhibited by digitonin and polyanethol sulphonate and it was concluded that the species required sterol for growth.

The sensitivity to digitonin shown by strains 4229 and B2/85 provided an indirect indication of their sterol requirement. The zones of inhibition were within the normal range of 3-12 mm seen with the sterol requiring families (Subcommittee on the Taxonomy of *Mollicutes*, 1979). The preliminary results suggested by the pilot test on agar were thus confirmed.

The untyped strains also exhibited sensitivity to SPS with inhibition zones of 5-6 mm. This was considered as a second indirect indication of their sterol requirement. Thus, it was concluded that both untyped strains require cholesterol for growth and they therefore belong to family *Mycoplasmataceae* and not to the non-sterol requiring *Acholeplasmataceae* .

Neither of the untyped strains hydrolysed urea using the broth method and the results were confirmed by the agar method. This indicated that the organisms do not belong to the genus *Ureaplasma*. In the agar method incubation up to 5 days resulted in only a weak reaction with the positive control organism, and this was thought to be due to depletion of the urea incorporated in the test medium by the organisms and dissipation into the atmosphere of the ammonia liberated by urea hydrolysis.

The remaining biochemical tests together with serological tests were carried out to provide the species description.

The tests for glucose breakdown and hydrolysis of arginine indicated that the untyped strains were able to utilize glucose but unable to hydrolyse arginine. In the test for glucose breakdown using the method of Aluotto *et al.* (1970) it has been found important to include certain controls in addition to the known positive and negative culture controls. These include an inoculated broth without glucose, since it has been found that a fall in pH of the medium does not necessarily indicate utilization of glucose, but might be due to metabolism of compounds other than glucose. This has been observed with non-fermenting mycoplasmas such as *M.bovigenitalium, M.bovis* and *M.agalactiae* (Edward and Moore, 1975). In addition, the basal medium may contain traces of glucose and arginine and it is then recommended to pretreat the basal medium with glucose oxidase, peroxidase, and arginine decarboxylase to remove any of traces of these substances (Freundt *et al.* 1979). Moreover, a slight fall in pH may be observed in uninoculated medium after several days of incubation due to presence of yeast extract and it is therefore essential to include an uninoculated control in the test.

In these studies all the controls were satisfactory and the results were clear-cut, but the glucose utilization was confirmed by a second method which determines directly the glucose disappearance from the growth medium. Both tests indicated that strain B2/85 depleted glucose more rapidly than strain 4229.

The results of the phosphatase test were interpreted as negative for strains 4229 and B2/85 since no pink colour was seen after 3 or 7 days incubation. The weak positive reactions that were seen after 14 days also occurred in the uninoculated control plates. These weak reactions might be due to the presence of uninactivated phosphatase or spontaneous degradation of sodium phenolphthalein diphosphate in

the medium. The agar plate test may be less satisfactory than the broth test, in which the culture medium is removed before addition of the substrate, and which uses only a short (4 hour) incubation time (Freundt *et al.* 1979).

The untyped strains were unable to liquefy coagulated serum. Accumulated moisture was seen in all the tubes including the controls and was probably due to the use of screw caps on the tubes instead of the cotton wool plugs described in the original method (Aluotto *et al.* 1970). Therefore only the presence of pits or depressions in the surface of the medium was interpreted as evidence of liquefaction (Freundt *et al.* 1979).

Neither strain 4229 nor strain B2/85 was capable of producing a film and spots reaction. The test for triphenyltetrazolium reduction indicated that both strains have oxidative enzyme activitiy capable of reducing the substrate under both aerobic and anaerobic incubation conditions.

The inability of strains 4229 and B2/85 to hydrolyse aesculin and arbutin indicated the absence of ß-D glucosidase activity in these organisms. This was the expected result since this enzyme activity has so far been found only in some *Acholeplasma* species (Williams and Wittler, 1971; Rose and Tully, 1983).

Strains 4229 and B2/85 both gave a haemadsorption reaction with red cells of chicken, turkey and duck. Manchee and Taylor-Robinson (1968) reported that colonies of all of eight strains of *M.gallisepticum* haemadsorbed red cells although not all the strains of other test species did. In the present study, the haemadsorption reaction of strains 4229 and B2/85 was of similar intensity to that seen with *M.gallisepticum* S6LP. In our studies only avian red cells from chicken, turkey and duck were tested. Variation in the ability to haemadsorb erthrocytes from different hosts has been reported (Gardella and DelGiudice, 1983). With *M.pulmonis* the

source of red blood cells was found to greatly influence the results and even the strain of donor mouse could affect results with the same mycoplasma strain (Davidson et al., 1988).

Strains 4229 and B2/85 both haemagglutinated chicken red cells in a similar manner to the reaction with *M.gallisepticum* with HA reciprocal titres of 16 or 32. Dupiellet (1988) found that strain 4229 and the similar goose strains haemagglutinated red cells from sheep, chicken, turkey goose and from Barbary, Pekin and 'mule' ducks. However, they found that these strains gave considerably higher haemagglutination titres than we obtained, and also higher titres than *M.gallisepticum* S6 and PG31. The higher titres with strain 4229 could possibly be explained by the methods of preparation of antigen. The antigen prepared in our study was concentrated 100 fold whereas it was concentrated 500 fold in the method of Dupiellet (1988).

Although strains 4229 and B2/85 show both HAd and HA, lack of correlation has been demonstrated between these phenomena. *M.gallisepticum* is one of the species which has both properties while other species such as *M.pulmonis* show only haemadsorption and still other mycoplasma species such as *M.neurolyticum* haemagglutinate only (Manchee and Taylor-Robinson, 1968). On the other hand, a correlation between HA and HAd of *M.synoviae* strains was observed by Rhoades (1985) where adsorption of red cells to colonies was directly correlated with HA activity.

The determination of the nucleic acid base composition is one of the four major trends for nucleic acid studies that have been considered relevant to mollicute taxonomy. The others are estimation of the genome size, determination of the nucleic acid homologies by DNA:DNA hybridization, and the determination of the electrophoretic mobility of ribosomal RNAs (Freundt and Edward, 1979). As

discussed in Chapter 1, the usefulness of genome size in determining the higher taxa of *Mollicutes* is now in question.

Determination of the guanine plus cytosine (G+C) content of the DNA is recommended for description of a new species by the Subcommittee on the Taxonomy of *Mollicutes* (1979) and this was reported for strain 4229 and three goose strains by Dupiellet (1988) and Dupiellet *et al.* (1990) by both melting temperature (Tm) and buoyant density methods. There was good agreement between the results of the two methods giving G+C contents of 31-32 mol% for the four strains. These were lower by approximately 1-2% than the values obtained for *M.gallisepticum* PG31 and S6. The DNA base composition of strain B2/85 has not been determined but for strain 4229 was within the range known for the mycoplasma group (23-41 mol%) (Stanbridge and Reff, 1979; Subcommittee on the Taxonomy of *Mollicutes*, 1979).

A summary of all the biochemical test results for strains 4229, B2/85 and *M.gallisepticum* is given in Table 5:13. No biochemical differences were found between these organisms.

Chapter 6 Serological characterization

Chapter 6 Serological characterization

6.1 Introduction

In organisms that reproduce sexually a species is defined by the ability of its members to breed with one another, but for those organisms that reproduce asexually the concept of species exhibits a problem which has not yet reached a satisfactory solution (Freundt *et al.*, 1979). The concept of species in all prokaryotic organisms, including those which belong to the class *Mollicutes*, is an arbitrary one for convenient designation of strains. In traditional microbial taxonomy, the grouping of strains was based mainly on related phenotypic characters, but mycoplasmas have relatively few distinctive morphological, cultural and biochemical properties, making this approach difficult.

The species definition of *Mollicutes* as defined by Subcommittee on Taxonomy of the *Mollicutes*, (1979) is ideally a cluster of morphologically similar isolates whose genome exhibits a high degree of relatedness, and the development of nucleic acid homology techniques has provided the means for a genetic approach to classification of these microorganisms. However, at present, extensive DNA hydridization studies between strains is not a feasible approach to identification and classification of mollicute species for most laboratories.

Historically the mollicute species have been defined as strains or groups of strains showing consistent and significant serological distinctness from other strains. Thus identification of an unknown strain at the species level depends for the final classification on serological relatedness (Subcommittee on the Taxonomy of the *Mollicutes*, 1979 ; Freundt *et al.*, 1979).

Strain 4229 has shown a serological relationship with *M.gallisepticum*, and was originally identified as that species, as were related strains (20807, 30902 and 35105) isolated from geese (Buntze *et al.*, 1986; Dupiellet 1988; Dupiellet *et al.*, 1990; Bradbury, personal communication). However, since DNA:DNA hybridization studies revealed only an approximate 40% relationship at the genomic level between strain 4229 and *M.gallisepticum* (Dupiellet *et al.*, 1990) it was considered that the two organisms belong to separate species (Johnson, 1984; *Ad Hoc* Committee of the International Committee on Systematic Bacteriology, 1987). This in turn raised the possibility that strain 4229 and the closely related strains represent a new species. The serological studies in this Chapter were carried out to determine if the untyped strains 4229 and B2/85 are serologically distinct from all the other recognised avian and mammalian *Mycoplasma* species and can therefore be proposed as a new species of *Mycoplasma*..

At least two serological tests are recommended by the Subcommittee on the Taxonomy of the *Mollicutes* (1979) for establishing new species, and as a minimum the organism should be cross-tested with all recognized species of the genus.

The serological tests commonly used vary in specificity and sensitivity. A specific test has been defined as one that will identify an organism as belonging to a certain species without revealing a significant degree of cross-reaction with representatives of any other species, whereas a sensitive test has been described as one giving a positive reaction with a small amount of antibody (Freundt *et al.*, 1979).

The recommended tests for distinguishing proposed new species of *Mycoplasma* are usually the growth inhibition (GI) test (Clyde, 1964) and immunofluorescence (DelGiudice *et al.*, 1967; Rosendal and Black, 1972). The GI test has a low degree of sensitivity but good specificity while immunofluorescence is both specific and sensitive (Freundt *et al.*, 1979). The latter test is highly reliable, not expensive with

regard to reagents, and gives results within a few hours. On the other hand both tests occasionally exhibit cross-reactions between different species of mycoplasmas or acholeplasmas (Freundt, *et al.*, 1979; Jordan *et al.*, 1982). The GI test and indirect immunofluorescence were the tests selected for these studies. The metabolism inhibition test is another alternative. It is less easy to perform but may be useful in clarifying any cross reactions seen in the other tests.

6.2 Materials and methods

6.2.1 Rabbit antisera

The sources of reference antisera are given in 2.7. Antisera against strains 1220, 1223, 19756 and 700 had been prepared in rabbits by other workers in our laboratory by the method given below. Antisera against strains 4229 and B2/85 were prepared as part of this study by the same method.

Procedure:

Strains 4229 and B2/85 (three times cloned) were adapted to serum fraction broth (SFB) using three to four passages. A culture of each strain was then grown up in 2 litres of SFB for 48 hours at 37°C and harvested by centrifugation at 2,000 g for 1.5 hours at 8°C. The supernatant was discarded and the sedimented pellet washed twice in 20 ml PBS pH 7.0. The final pellet was resuspended in 10 ml PBS, mixed well on a vortex mixer and a sample was inoculated onto BA to check for contamination. An aliquot was removed for estimation of protein concentration using the Pierce Bicinchoninic acid (BCA) protein assay reagent. Antigen preparations were stored at -20°C.

The antigen was prepared for administration to rabbits in three forms: unadjuvanted, adjuvanted with Freund's Complete Adjuvant[1] and adjuvanted with Freund's Incomplete Adjuvant[1]. The non-adjuvanted antigen was adjusted with PBS to contain 5 mg protein/ml.

For the adjuvanted preparations the antigen suspension was emulsified with an equal volume of the appropriate adjuvant to give a final concentration of 5 mg/ml using a Polytron[2] homogenizer. The stability of the emulsified antigen was tested by placing a small droplet on the surface of water and examining for dispersion. If the drop remained discrete the antigen was used for injection of the rabbits using sterile glass syringes with 23-gauge needles. If dispersion occurred, further emulsification was carried out.

Inoculation schedule

Two Dutch white rabbits were used for each strain. Each was pre-bled from the marginal ear vein and then given two intramuscular and two subcutaneous inoculations, each containing 2.5 mg of antigen protein in Freund's Complete Adjuvant. Each rabbit also received an intravenous injection containing 5 mg of non-adjuvanted antigen protein.

At 21 days a booster intradermal inoculation of 5 mg of antigen protein in Freund's Incomplete Adjuvant was given to each rabbit . A test bleed was carried out after seven to ten days. The serum was immediately tested by immmunofluorescence and growth inhibition test, and, if the results were satisfactory, the rabbits were exsanguinated.

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1 Difco Laboratories, Detroit, Michigan, U.S.A

2 The North Media Supply Ltd., Blanket Row, Hull, U.K.

The serum was inactivated at 56°C for 30 minutes and stored at -20°C.

6.2.2 Indirect immunofluorescence test

The immunofluorescence technique was first developed by Coons *et al.* (1941) using fluorescent dyes such as fluorescein or rhodamine, coupled with antibodies, which emit fluorescence when irradiated with ultra-violet light. Two methods are basically used, the direct and indirect methods. In the former antibodies are conjugated with a fluorescent dye and allowed to react with the antigen. If the antibodies are specific to the antigen they will attach and can be seen to emit fluorescence when viewed on a fluorescence microscope illuminated by light of a suitable wavelength. In the latter method, unconjugated specific antiserum is allowed to react with antigen and then a labelled antiglobulin conjugate is added.

Immunofluorescence was introduced to mycoplasmology by Liu (1957) who used it for demonstration of *M.pneumoniae* in chick embryos. Later, Chanock *et al.* (1962) used the technique to identify *M.pneumoniae* by transferring colonies onto microscope slides. This technique was somewhat laborious and an advance came when DelGiudice *et al.* (1967) developed epi-immunofluorescence, using the microscope objective lens to serve also as condenser for incident illumination. This allowed the application of the fluorescent antibody staining method to mycoplasma colonies *in situ* and has subsequently proved to be of great value, particularly for its ability detect mixed cultures (Del Guidice *et al.*, 1967; Al Aubaidi and Fabricant, 1971; Bradbury, 1982).

The technique chosen for the present study was the indirect fluorescent antibody (IFA) test using colony bearing agar blocks as described by Rosendal and Black (1972). This method has the advantage that only one fluorescent conjugate is needed and it is available commercially. This is in contrast to the direct test which

requires conjugates to be prepared for each mycoplasma species to be identified. The indirect test is more sensitive than the direct test and therefore requires a smaller amount of antiserum. It is also less prone to non-specific background fluorescence, which can interfere with reading the results and interpretation (Freundt *et al.*, 1979).

Fluorescence microscopy

IFA tests were examined with a Leitz Ortholux microscope[1]. It was fitted with an ultra-high pressure mercury vapour lamp which emitted incident ultra-violet illumination, a dichroic beam splitting mirror with built-in suppression filters (TK 510/ K515) and an exciter filter (CB 16.5). A coloured glass filter (BG 38) was included in the lamp-housing to prevent low level transmission in the red spectrum from becoming visible.

Materials:

Antisera

Rabbit antisera were diluted 1/40 in PBS, pH 7.0 and stored at -20°C.

Conjugate

Commercial goat anti-rabbit globulin[2] (F9262; IgG fraction of antiserum to rabbit IgG, whole molecule) conjugated with FITC was reconstituted in distilled water, diluted 1/60 in PBS, pH 7.0 and stored at 4°C for up to one week.

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1 E. Leitz (Instruments) Ltd., London, U.K..

2 Sigma Chemical Chemical Company Ltd, Poole, U.K.

Procedure:

Agar plates were inoculated with broth cultures containing approximately 10^3 - 10^4 CFU/ml and incubated at 37°C until small discrete colonies had developed. Rectangular colony-bearing blocks (1.0 x 0.5 cm) were cut with a scalpel and the lower right hand corner was cut off to facilitate orientation. The blocks, with their colonies uppermost, were placed on labelled glass microscope slides and 20 µl of the appropriate diluted antiserum was added. The blocks were incubated in a humid chamber at room temperature for 30 minutes and then transferred to individual labelled tubes containing 10 ml PBS, pH 7.0. The tubes were plugged with rubber stoppers and placed on an automatic test tube rotator[1] for 10 minutes. The PBS in each tube was discarded into a beaker containing a 1% (v/v) solution of FAM[2], and a further 10 ml PBS was added. The washing step was repeated and then the blocks were placed back on their original microscope slides, ensuring that they were in the correct orientation. After the excess moisture had evaporated 10 µl of diluted commercial goat anti-rabbit conjugate was added to each agar block and the above incubation and washing procedure was repeated. The blocks were then placed back on the slides and examined under the fluorescence microscope.

If at any stage a substantial interruption in the procedure occurred, the blocks were left submerged in PBS at 4°C. Homologous and heterologous control cultures were included in each series of tests, and colonies of the test strain was also tested with normal rabbit serum diluted at 1/40 in PBS in order to control for non-specific fluorescence. In a positive reaction the colonies exhibited apple-green fluorescence whereas negative colonies exhibited dull dark green or a yellow/green glow.

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1 Anderman Company Ltd., Surrey, U.K.

2 Evans Vanodine International Ltd., Preston, U.K

The fluorescence was graded on an arbitrary scale as follows:

3+ = very bright apple green

2+ = bright apple green

1+ = apple green

gl = glow - colonies tinged yellow/green (not regarded as a positive result)

0 = no fluorescence

Investigations:

Evaluation of rabbit antisera to strains 4229 and B2/85

The rabbit antisera were titrated with the homologous and the heterologous strain. Endpoints were taken as the highest dilution giving a positive reaction.

Cross-testing of rabbit antisera with *M.gallisepticum*

One rabbit antiserum to 4229 and one to B2/85 were selected on the basis of their GI reactions and cross tested with each other and with *M.gallisepticum* S6LP. In addition a rabbit antiserum to strain 4229 prepared by Dupiellet *et al.*, (1990) was included for comparison. Reference antiserum to *M.gallisepticum* PG31 was also titrated against the homologous strain, S6LP, 4229 and B2/85.

Tests with avian *Mycoplasma* species and serovars

The two untyped strains were compared with all the established avian *Mycoplasma* species and serovars and with four other potential new avian species. The tests were performed in two ways: with cultures of the untyped strains and the avian reference antisera and with the avian reference cultures and antisera prepared against the untyped strains.

Tests with mammalian *Mycoplasma* species

The two untyped strains were tested against antisera to the established mammalian reference strains. In cases where we were unable to grow the reference culture or not permitted to do so (*M.mycoides* subsp *mycoides* and *M.mycoides* subsp *capri*) the tests were performed without homologous positive controls.

6.2.3 Growth inhibition test

The growth inhibition test was first introduced by Nicol and Edward (1953) who observed that antiserum included in agar medium inhibited the growth of mycoplasma colonies of homologous and closely related strains. Later on, Edward and Fitzgerald (1954) were the first to exploit the method for serological identification of the mycoplasma species.

Having the advantage of high specificity, ease of performance and economy, the test has been extensively used for serological identification of mycoplasmas (Freundt *et al.*, 1973; Clyde, 1983).

The mechanism of growth inhibition is not fully known, but various theories have been suggested. One possibility is that, because of the lack of a cell wall the transport systems of the cell membrane are blocked (Edward and Fitzgerald, 1954). Another theory is that there is complement-dependent lysis of the mycoplasma cell (Roberts, 1971) but further studies were needed to elucidate the mechamisms involved.

The original method has been replaced by a number of modifications introduced in order to reduce the amount of antiserum as well as to increase the sensitivity of the test (Clyde, 1964 ; Black, 1973 ; Freundt *et al.*, 1973). The method described by

Clyde (1964) which is performed using antiserum-impregnated paper discs has been widely adopted and the modification described by Black (1973) in which wells are made in the agar and filled with antiserum was used in the present study.

Materials:

Media

The medium used for almost all *Mycoplasma* species was MA using 7 ml in Petri dishes of 5 cm diameter, giving a depth of approximately 4 mm. Species requiring other growth media were also tested on 7 ml agar plates of the appropriate medium (2.3).

Antisera

Undiluted rabbit antisera were used.

Procedure:

Two different dilutions of the test organisms were prepared in MB with the aim of obtaining an inoculum containing about 10^5 CFU/ml, this being the optimum concentration for growth inhibition (Freundt *et al.*, 1979).

A predried agar plate was inoculated with both dilutions of the test organism with a platinum loop and the running drop method, and the centre of each inoculated run was marked on the underside of the plate. After absorption of the inoculum, a well of 4 mm diameter was punched in the agar at the marked spot with a sterile stainless steel cylinder. The agar plug was removed and the resulting well was filled with approximately 40 µl antiserum.

After the antiserum had absorbed, the plates were inverted and incubated in candle jars at 27°C for 48 hours. They were then incubated at 37°C and examined daily with a stereomicroscope until colonies were visible. Those *Mycoplasma* species that required a specific gaseous environment (2.5) were incubated in Gas Pak jars. Homologous controls were included in the tests.

The zone of colony inhibition was measured in mm from the edge of the well to the edge of the area of colony growth. The occurrence of breakthrough colonies within the inhibition zone was recorded.

Investigations:

Evaluation of rabbit antisera to strains 4229 and B2/85

The sera from the four rabbits were tested against the homologous strains in order to select the best of each for subsequent use.

Tests with avian *Mycoplasma* species and serovars

The two untyped strains were tested by GI with all the established avian *Mycoplasma* species and serovars, and with the four other potential new species. The tests were performed in two ways: with the untyped strains and the avian reference antisera and with the avian reference cultures and antisera prepared against the untyped strains.

Tests with mammalian mycoplasma species

Almost all the established mammalian reference mycoplasma strains were tested against antisera prepared against the two untyped strains using the GI test. For

those species that we were unable to grow the GI tests were performed using the reference antisera to them and cultures of 4229 and B2/85, but no positive controls could be included.

6.3 Results

6.3.1 Indirect immunofluorescence test

Evaluation of rabbit antisera to 4229 and B2/85

The serum from the test bleeds of all 4 rabbits gave IFA titres of more than 1 in 1280 with the homologous and heterologous strain. These were considered high enough to proceed with exsanguination of the rabbits.

Cross-testing of rabbit antisera with *M.gallisepticum*

The results are shown on Table 6:1. Antisera to strains 4229 and B2/85 gave high titres in the homologous tests and when cross-tested with each other. The antisera to 4229 and to B2/85 prepared by us gave titres of 1 in 320 with M.*gallisepticum* S6LP but only 1 in 40 with the type strain PG31. That prepared by Dupiellet *et al.* (1990) had lower cross-reactions with these two strains. The reference antiserum to PG31 reacted strongly with strains 4229 and B2/85.

Tests with avian *Mycoplasma* species and serovars

The results of immunofluorescence tests between the untyped strains and the avian reference strains are shown in Tables 6:2 and 6:3. The positive controls all gave a

Table 6:1 **Cross-testing of untyped strains and *M.gallisepticum* by immunofluorescence**

	Antisera			
Strains	4229[1]	4229	B2/85	PG31
4229	640[2]	1280	2560	320
B2/85	640	1280	1280	160
PG31	20	40	40	>1280
S6LP	40	320	320	>1280

1. This antiserum was prepared by Dupiellet *et al.*, (1990)
2. Reciprocal titre.

Table 6:2 Immunofluorescence tests between strains 4229 and B2/85 and avian reference antisera

Antiserum	Homologous reference control	Culture	
		4229	B2/85
4229		3+[1]	3+
B2/85		3+	3+
M.anatis 1340	3+	0	0
M.anseris 1219	2+	0	0
M.cloacale 383	2+	gl[2]	gl
M.columbinasale 694	1+	0	0
M.columbinum MMP1	2+	0	0
M.columborale MMP4	1+	0	0
M.gallinaceum DD	2+	0	0
M.gallinarum PG16	2+	0	0
M.gallisepticum PG31	3+	1+	1+
M.gallopavonis WR1	2+	gl	0
M.glycophilum 486	2+	gl	0
M.iners PG30	2+	0	0
M.iowae 695	3+	0	0
M.lipofaciens R171	2+	0	0
M.meleagridis 17529	2+	0	0
M.pullorum CKK	3+	0	0
M.synoviae WVU1853	3+	0	0
Avian serovars:			
J strain DJA	2+	gl	gl
K strain DK-CPA	2+	gl	gl
N strain FMN	1+	0	0
Q strain L3-10B	2+	gl	gl
R strain DRA-O	1+	0	0
Untyped avian strains:			
1220	3+	0	0
1223	3+	0	0
19756	3+	0	0
700	3+	0	0

1. Fluorescence was graded on a scale of 0 to 3+ (6.2.2)
2. Glowing reaction, not regarded as positive

Table 6:3 Immunofluorescence tests between antisera to strains 4229 and B2/85 and avian reference cultures

Cultures	Antiserum	
	4229	B2/85
M.anatis 1340	0[1]	gl[2]
M.anseris 1219	0	0
M.cloacale 383	0	0
M.columbinasale 694	0	0
M.columbinum MMP1	gl	gl
M.columborale MMP4	0	0
M.gallinaceum DD	0	0
M.gallinarum PG16	0	0
M.gallisepticum PG31	1+	1+
M.gallopavonis WR1	0	0
M.glycophilum 486	0	0
M.iners PG30	0	0
M.iowae 695	0	0
M.lipofaciens R171	gl	gl
M.meleagridis 17529	0	0
M.pullorum CKK	0	0
M.synoviae WVU1853	0	0
Avian serovars:		
J strain DJA	0	0
K strain DK-CPA	0	0
N strain FMN	0	0
Q strain L3-10B	0	0
R strain DRA-O	0	0
Untyped avian strains:		
1220	0	0
1223	0	0
19756	0	0
700	gl	0

1. Fluorescence was graded on a scale of 0 to 3+ (6.2.2)
2. Glowing reaction, not regarded as positive

satisfactory fluorescence reaction. The normal rabbit serum controls were all negative although a few glowing reactions were recorded.

In the tests performed between the untyped cultures and antisera to the avian reference strains, no positive reactions were seen except with *M.gallisepticum*. This occurred with both untyped strains but the fluorescence was less intense than the homologous reaction (Table 6:2). A green glow was recorded in a number of tests with both untyped strains but none was strong enough to be considered positive.

In reciprocal tests between the antisera prepared against the untyped strains and the avian reference cultures, no positive reactions comparable with homologous controls were exhibited except that a positive reaction was seen again between *M.gallisepticum* and strains 4229 and B2/85 (Table 6:3). A green glow was also seen on a number of occasions.

Tests with mammalian *Mycoplasma* species

The results of tests using antisera to the mammalian mycoplasma reference strains and the two untyped strains are shown in Table 6:4. The homologous controls for the reference mammalian species showed a positive fluorescence reaction. No fluorescence was seen in the normal rabbit serum controls although a number of a green glowing reactions were recorded in these tests and those with the reference antisera. None of them were regarded as a positive reaction.

Table 6:4 Immunofluorescence tests between strains 4229 and B2/85 and mammalian reference antisera

Antiserum	Homologous reference control	Culture	
		4229	B2/85
M.agalactiae PG2	2+[1]	0	0
M.alkalescens D12	3+	0	0
M.alvi Ilsley	2+	0	0
M.arginini G230	3+	0	0
M.arthritidis PG6	1+	0	0
M.bovigenitalium PG11	2+	0	0
M.bovirhinis PG43	3+	0	0
M.bovis Donetta	1+	gl[2]	gl
M.bovoculi M165/69	1+	gl	gl
M.buccale CH20247	1+	0	0
M.californicum ST-6	2+	0	0
M.canadense 275C	2+	0	0
M.canis PG14	1+	0	0
M.capricolum (California Kid)	1+	0	0
M.caviae G122	3+	0	0
M.cavipharyngis 117C	3+	0	0
M.citelli RG-2C	3+	0	0
M.collis 58B	2+	0	0
M.conjunctivae HRC581	3+	0	0
M.cricetuli CH	2+	0	0
M.cynos H831	3+	0	0
M.dispar 462/2	2+	0	0
M.edwardii PG24	2+	0	0
M.ellychniae ELCN-1	*[3]	gl	0
M.equigenitalium T37	2+	0	0
M.equirhinis M432/72	2+	0	0
M.fastidiosum 4822	*	0	0
M.faucium DC333	2+	gl	gl
M.felifaucium PU	2+	0	0
M.feliminutum Ben	3+	0	0
M.felis CO	3+	0	0
M.fermentans PG18	3+	0	0
M.flocculare Ms42	2+	0	0
M.gateae CS	3+	0	0

(continued on next page)

(Table 6:4 continued)

Antiserum	Homologous reference control	Culture	
		4229	B2/85
M.genitalium G37	*	gl	0
M.hominis PG21	3+	0	0
M.hyopneumoniae J	*	gl	gl
M.hyorhinis BTS-7	3+	0	0
M.hyopharyngis H3-6B F	2+	0	gl
M.hyosynoviae S16	*	gl	gl
M.lipophilum MaBy	3+	gl	0
M.maculosum PG15	2+	0	0
M.moatsii MK405	3+	0	0
M.mobile 163K	3+	gl	0
M.molare H542	2+	0	0
M.muris RIII4	2+	gl	gl
M.mustelae MX9	2+	0	0
M.mycoides subsp. *capri* PG3	*	0	0
M.mycoides subsp. *mycoides* PG1	*	0	0
M.neurolyticum A	3+	0	0
M.opalescens MH5408	3+	0	0
M.orale CH19299	3+	0	0
M.ovipneumoniae Y-98	3+	0	0
M.pirum 70-159	3+	0	0
M.pneumoniae FH	3+	gl	gl
M.primatum HRC292	3+	0	0
M.pulmonis PG34	3+	0	0
M.putrefaciens KS1+1	3+	0	0
M.salivarium PG20	3+	0	0
M.spumans PG13	3+	0	0
M.sualvi Mayfield B	3+	gl	0
M.subdolum TB	3+	0	0
M.testudinis 01008	3+	0	0
M.verecundum 107	3+	0	0

1. Fluorescence was graded on a scale of 0 to 3+ (6.2.2)
2. Glowing reaction, not regarded as positive
3. Tests performed without homologous controls

6.3.2 Growth inhibition test

Tests with avian *Mycoplasma* species and serovars

The inhibition zones produced in the test with homologous strains and their antisera were satisfactory, ranging between 5 and 7 mm. No growth inhibition reactions were seen between cultures of strains 4229 or B2/85 and antisera to the avian reference strains (Table 6:5) with the exception of a small inhibition zone (1 mm) seen with antiserum against avian serovar J (strain DJA).

No inhibition was observed with antisera prepared against the untyped strains in tests against the avian reference cultures (Table 6:6) with the exception of *M.gallisepticum* culture. This mycoplasma showed small inhibition zones (1mm and 2mm) with antisera against strain 4229 and B2/85 respectively.

Tests with mammalian *Mycoplasma* species

The results of growth inhibition between antisera prepared against untyped strains and the mammalian reference cultures are shown in Table 6:7. Inhibition reactions were not observed with any of the mammalian reference cultures.

6.4 Discussion

The serological relationship between strain 4229 and *M.gallisepticum* that was suggested by our original diagnostic immunofluorescence tests and then observed by Dupiellet *et al.* (1990) using GI and metabolism inhibition tests, has been confirmed by immunofluorescence cross-testing here.

We have also shown strain B2/85 to be closely related serologically to strain 4229. This supports the results of genetic studies by Yogev *et al.* (1988a; b) using the

Table 6:5 Growth inhibition tests between strains 4229 and B2/85 and avian reference antisera

Antiserum	Homologous reference control	Culture	
		4229	B2/85
4229		6[1]	5
B2/85[2]		5	7
M.anatis 1340	10	0	0
M.anseris 1219	7	0	0
M.cloacale 383	6	0	0
M.columbinasale 694	6	0	0
M.columbinum MMP1	3	0	0
M.columborale MMP4	7	0	0
M.gallinaceum DD	2	0	0
M.gallinarum PG16	5	0	0
M.gallisepticum PG31	5	0	0
M.gallopavonis WR1	12	0	0
M.glycophilum 486	3	0	0
M.iners PG30	8	0	0
M.iowae 695	10	0	0
M.lipofaciens R171	12	0	0
M.meleagridis 17529	4	0	0
M.pullorum CKK	4	0	0
M.synoviae WVU1853	4	0	0
Avian serovars:			
J strain DJA	2	1	1
K strain DK-CPA	12	0	0
N strain FMN	12	0	0
Q strain L3-10B	6	0	0
R strain DRA-O	8	0	0
Untyped avian strains:			
1220	10	0	0
1223	9	0	0
19756	7	0	0
700	6[2]	0	0

1. Size of inhibition zone in mm
2. Breakthrough colonies seen

Table 6:6 Growth inhibition tests between antisera to strains 4229 and B2/85 and avian reference cultures

Culture	Antiserum	
	4229	B2/85
M.anatis 1340	0[1]	0
M.anseris 1219	0	0
M.cloacale 383	0	0
M.columbinasale 694	0	0
M.columbinum MMP1	0	0
M.columborale MMP4	0	0
M.gallinaceum DD	0	0
M.gallinarum PG16	0	0
M.gallisepticum PG31	1	2
M.gallopavoni s WR1	0	0
M.glycophilum 486	0	0
M.iners PG30	0	0
M.iowae 695	0	0
M.lipofaciens	0	0
M.meleagridis 17529	0	0
M.pullorum CKK	0	0
M.synoviae WVU1853	0	0
Avian serovars:		
J strain DJA	0	0
K strain DK-CPA	0	0
N strain FMN	0	0
Q strain L3-10B	0	0
R strain DRA-O	0	0
Untyped avian strains:		
1220	0	0
1223	0	0
19756	0	0
700	0	0

1. Size of inhibition zone in mm

Table 6:7 Growth inhibition tests between antisera to strains 4229 and B2/85 and mammalian reference cultures

Culture	Homologous control	Antiserum	
		4229	B2/85
M.agalactiae PG2	4[1]	0	0
M.alkalescens D12	3[2]	0	0
M.alvi Ilsley	4	0	0
M.arginini G230	7	0	0
M.arthritidis PG6	7	0	0
M.bovigenitalium PG11	7	0	0
M.bovirhinis PG43	3	0	0
M.bovis Donetta	3	0	0
M.bovoculi M165/69	5	0	0
M.buccale CH20247	5	0	0
M.californicum ST-6	6	0	0
M.canadense 275C	8	0	0
M.canis PG14	3	0	0
M.capricolum California Kid	5	0	0
M.caviae G122	9	0	0
M.cavipharyngis 117C	*	0	0
M.citelli RG-2C	9	0	0
M.collis 58B	9	0	0
M.conjunctivae HRC581	4[2]	0	0
M.cricetuli CH	6	0	0
M.cynos H831	3[2]	0	0
M.dispar 462/2	*[3]	0	0
M.edwardii PG24	10	0	0
M.ellychniae ELCN-1	*	0	0
M.equigenitalium T37	8	0	0
M.equirhinis M432/72	*	0	0
M.fastidiosum 4822	*	0	0
M.faucium DC333	*	0	0
M.felifaucium PU	10	0	0
M.feliminutum Ben	*	0	0
M.felis CO	15	0	0
M.fermentans PG18	3	0	0
M.flocculare Ms42	*	0	0
M.gateae CS	7	0	0
M.genitalium G37	*	0	0

(continued on next page)

(Table 6:7 continued)

Culture		Antiserum	
		4229	B2/85
M.hominis PG21	5	0	0
M.hyopneumoniae J	*	0	0
M.hyorhinis BTS-7	2[2]	0	0
M.hyopharyngis H3-6B F	4	0	0
M.hyosynoviae S16	*	0	0
M.lipophilum MaBy	3[2]	0	0
M.maculosum PG15	7	0	0
M.moatsii MK405	10	0	0
M.mobile 163K	2[2]	0	0
M.molare H542	4	0	0
M.muris RIII4	7	0	0
M.mustelae MX9	4[2]	0	0
M.mycoides subsp. *capri* PG3	*	0	0
M.mycoides subsp. *mycoides* PG1	*	0	0
M.neurolyticum A	7	0	0
M.opalescens MH5408	6[2]	0	0
M.orale CH19299	11	0	1
M.ovipneumoniae Y-98	10	0	0
M.pirum 70-159	5	0	0
M.pneumoniae FH	3[2]	0	0
M.primatum HRC292	6	0	0
M.pulmonis PG34	*	0	0
M.putrefaciens KS-1	5	0	0
M.salivarium PG20	12	0	0
M.spumans PG13	6	0	0
M.suaivi Mayfield B	8	0	0
M.subdolum TB	9	0	0
M.testudinis 01008	*	0	0
M.verecundum 107	3	0	0
4229		6	
B2/85			7

1 Size of inhibition zone in mm
2. Breakthrough colonies seen
3. Tests were performed without a positive control and using the mammalian reference antisera with cultures of 4229 and B2/85

rRNA and *tuf* gene probes to analyse both these strains, and is also in accord with the similarities shown by the restriction enzyme analyses of their DNA in Chapter 3.

In the cross-testing by immunofluorescence of strains 4229 and B2/85 with the recognised *Mycoplasma* species the only one to show any significant reaction was the anticipated one with *M.gallisepticum*. A number of yellow/green glowing reactions were also recorded but they did not show the apple green fluoresence of a positive test. The agar block technique of immunofluorescence used here is considered to give fewer non-specific reactions than hot-water fixed colonies (Rosendal and Black, 1972). The reason for these glowing reactions is not known but could be due to non-specific attachment of serum or conjugate, or insufficient washing.

Ideally all the anti-mycoplasma sera should have been chessboard-titrated with the conjugate since it is unlikely that a single dilution of antiserum and conjugate will be the optimal for all (Erno, 1977). False negative results could occur if the reagents are too dilute, or even where they are too concentrated as a result of a prozone reaction. On the other hand false positive results might be obtained due to non-specific or minor interspecies antigenic relationships (Erno, 1977). The procedure of titration is time consuming and results obtained are complex so that it was not considered practical to undertake this task. Dilutions of conjugate and antiserum were standardised to 1/60 and 1/40 respectively and the positive controls that were included with almost all tests demonstrated that the reagents were satisfactory at these dilutions.

The antisera prepared in rabbits against strains 4229 and B2/85 were of adequate potency for performing the growth inhibition test as an inhibitory zone of 2 mm has been proposed as a minimum for such tests (Erno and Jurmanova, 1973). The sensitivity of the test is improved by using suboptimal growth conditions such as

lowering the incubation temperature to 27°C for the first two days (Freundt *et al.*, 1979) and it can be further enhanced by using thinner agar plates (Jordan, 1973).

In the GI tests between cultures of the untyped strains and the avian reference sera there was only one reaction, which was with avian serovar J. This was seen with both untyped cultures but was not confirmed by the reciprocal tests or by immunofluorescence and was thus considered to be non-specific.

M.gallisepticum showed only a one-way cross-reaction in GI tests. Antiserum to strains 4229 and B2/85 gave inhibition zones of 1 and 2 mm respectively with the *M.gallisepticum* PG31 culture. Dupiellet *et al.* (1990) reported a two-way cross-reaction by GI test but it was thought in our case that the growth inhibiting potency of the PG31 antiserum may have declined during storage at -20°C (Bradbury, 1982).

GI tests using the antisera to the untyped strains with cultures of the established mammalian reference strains were all negative and confirmed the results of the immunofluorescence.

Since the work described above was completed, several new species of *Mycoplasma* have been described. These are *M.lucivorax*, *M.luminosum*, *M.somnilux* (Williamson *et al.*, 1990), *M.melaleucae* (Tully *et al.*, 1990), *M.lactucae* (Rose *et al.*, 1990) *M.oxoniensis* (Hill, 1991) *M.phocarhinis* and *M. phocacerebrale* (Giebel *et al.*, 1991). The type strains and antisera are being assembled and will be tested with strains 4229 and B2/85 in the near future. The descriptions of these species suggest that it is unlikely that any of them will prove to be closely related to our strains since their biochemical properties differ and none of them apparently possesses a terminal tip structure.

In conclusion, the results confirm the relationship of strains 4229 and B2/85 with *M.gallisepticum* and show that there is no relationship with the other recognised avian and mammalian *Mycoplasma* species. On the basis of these results, and the DNA:DNA hybridization data of Dupiellet *et al.* (1990) it appears that the two strains represent a new species of the genus *Mycoplasma*.

Since little is known about the pathogenicity of this organism, investigations were undertaken *in vitro* and *in vivo* and are described in the next two Chapters.

Chapter 7 Pathogenicity of strains 4229 and B2/85 in chick and duck embryo tracheal organ cultures

Chapter 7 Pathogenicity of strains 4229 and B2/85 in chick and duck embryo tracheal organ cultures

7.1 Growth and ciliostatic effects

7.1.1 Introduction

In the previous sections it was shown that strains 4229 and B2/85 are likely to represent a new species of the genus *Mycoplasma* and that the organism shares many similarities with the pathogenic avian species *M. gallisepticum.* These similarities include the possession of a terminal tip structure which, in the case of *M.gallisepticum* permits attachment of the organism to its target tissue and is regarded as the first step in the infection process (Razin, 1985).

In view of these observations it was of interest to investigate the likely pathogenicity of strains 4229 and B2/85. Some preliminary studies with strain 4429 were carried out by Buntze (1987) *in vivo* and by Dupiellet (1988) in cell cultures, *in ovo* and *in vivo*, but the use of organ cultures had not been investigated. The purpose of this section was to elucidate the growth and pathogenicity of strains 4229 and B2/85 in avian tracheal organ cultures.

Organ cultures have been described by Collier (1979) as 'models that permit 'the maintenance of complete rudiments or fragments of organs in a viable, differentiated, functional condition in a nutrient medium *in vitro*', whereas Gabridge (1979) defined organ or explant culture as 'the *in vitro* maintenance of a fragment of an organ having at least two major cell types and maintaining some degree of normalcy in regard to morphology, function and relative spatial relationships'.

Several experimental models have been used to study host-parasite interactions and tracheal organ culture (TOC) is one such model that has been used to monitor the interactions as well as to obtain knowledge about the cell-destroying capacities of the parasites. Many investigations have been performed with this model with viruses (e.g. Barski *et al.*, 1959; Bang and Niven, 1958; Reed, 1971, 1972; Westerberg, *et al.*, 1972), with bacteria (e.g. Baseman and Collier, 1974; Matsuyama, 1974; Murakami and Matsuyama, 1980; Bemis and Kennedy, 1981), and with mycoplasmas.

Several advantages of using suitable organ cultures over intact animals in pathogenicity studies have been described with reference to mycoplasma infections (Razin, 1978). The most important one is the ability to observe continually the effects of the parasite on its natural target cell under controlled conditions. In TOC ciliary activity continues for several days, or even weeks, permitting continuous monitoring of injury and viability of the epithelial cells. The system facilitates the study of toxic factors produced by the parasite and the metabolic disturbances caused by them. The pathogenicity of mixed mycoplasma and virus infections and their control by chemotherapy can also be conveniently studied using organ cultures, and finally, the use of organ cultures most probably eliminates hormonal effects and immunological responses as well as age, weight, and sex differences which are all factors that might affect the response of intact animal to the parasite. Although this simplifies the system, it is also the most serious deficiency, since it can argued that these factors, not functioning in organ cultures, might profoundly influence the response of the target cells to the parasite in the intact animal.

Thus, the organ culture system is convenient for study of the mechanisms of attachment and injury, but is not adequate to examine the immune response and repair process following infection because it does not possess a blood supply and

the elements of the immune system are not available to interact with infecting agents. As a result the injury process is probably magnified.

The first use of organ culture in studying the effects of mycoplasma on respiratory epithelium was in 1969 by Butler in England and by Collier (1969) in the United States. The former used tracheal cultures of human embryo for isolating and growing a number of mycoplasma species, and the latter inoculated *M.pneumoniae* into hamster TOCs. Since then there have been many reports of the study of mycoplasma infections in organ cultures prepared from tissues of various different host species.

Studies of the pathogenic effect of avian mycoplasmas on organ cultures have been most extensively performed using chicken TOCs. Cherry and Taylor-Robinson (1970a) were the first to describe the preparation and use of chicken TOCs for the study of mycoplasmas. Using this technique the growth and cilia-stopping effect of many mycoplasmas, including avian species, have been studied and it has been shown that some of them adversely affect ciliary activity. Examples include certain strains of *M.gallisepticum* (Cherry and Taylor-Robinson, 1970a, 1973; Butler and Ellaway, 1971; Abu-Zahr and Butler, 1976; Power and Jordan, 1976; Hirano, *et al.*, 1978; Takagi and Arakawa, 1980), *M.synoviae* (Butler and Ellaway, 1971; Hirano, *et al.*, 1978), and a strain of *M.meleagridis* (Cherry and Taylor-Robinson, 1973).

It has been found that the pathogenicity of *M. gallisepticum* for chick TOC is a useful guide to its pathogenicity in the host (Power and Jordan, 1976; Levisohn *et al.*, 1986). On the other hand *M.gallinarum*, which is of doubtful pathogenicity in chickens, does not impair the viability of chick embryo TOCs and may even enhance it (Power and Jordan, 1976; Cherry and Taylor-Robinson, 1970a; Taylor-Robinson and Cherry, 1972).

Thus it seemed to be a logical first step to study the effects of strains 4229 and B2/85 on the ciliary activity of chick and duck embryo TOCs. In order to obtain the maximum information on the host-parasite relationship and the possible function of the terminal tip structure, these studies were supplemented with immunofluorescence and transmission electron microscopy examinations of infected tissues.

7.1.2 Materials and Methods

Embryonated eggs

Fertile chicken eggs were obtained from the Department's specific pathogen-free (SPF) flock. The flock was kept under conditions of strict isolation, and was free of serum antibodies to the viruses of Newcastle disease, infectious bronchitis, infectious laryngotracheitis, Marek's disease, avian adeno- and reoviruses. In addition it was free of serum antibodies to *M.gallisepticum*, *M.synoviae*, *Salmonella pullorum* and *Salmonella gallinarum*.

Fertile duck eggs were obtained from a commercial company[1] and were from a flock of unknown mycoplasma status. On receipt these eggs were fumigated with formaldehyde gas. Both chicken and duck eggs were washed in a warm solution of mild detergent[2] and were stored for up to one week in a cool place. The eggs were set with the air space upwards in wooden trays then placed in an incubator[3] which maintained them at constant temperature (37°C) and humidity, and turned them at regular intervals.

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1 Cherry Valley Farms Ltd, Rothwell, Lincs, U.K.
2 Nusan, Coopers Animal Health Ltd, Crewe, U.K.
3 Western Incubators Ltd, Chelmsford, Essex, U.K.

Hepes buffer (N-2 hydroxy ethyl piperazine-N-2 ethanesulphonic acid)

This was prepared as a 0.05 M solution in distilled water, sterilised by autoclaving at 15 lb/sq.in for 15 minutes and stored at 4°C.

Organ culture medium (OCM)

The medium used was basically as described by Cherry and Taylor-Robinson, (1970a) but in these studies it was used both with and without a serum supplement.

To prepare 100 ml:	
Eagle's Minimal Essential Medium[1]	10.0 ml
(X10, + Earle's Salt + glutamine)	
Hepes buffer	85.0 ml
Penicillin	20,000 iu
Inactivated calf serum[2] (as appropriate)	5.0 ml

The pH was adjusted to 7.3 with M NaOH, the medium was sterilised by Seitz filtration and stored at 4°C.

The complete medium was tested for bacterial and fungal contamination before use by inoculating samples onto blood agar followed by incubation at 37°C, and onto Sabouraud Dextrose agar with incubation at 27°C (2.3). If there was no growth after 24 hours, the OCM was used, but as an extra precaution the plates were incubated for up to one week to ensure that any organ culture contamination did not originate from the medium. In addition, after the OCM was dispensed into sterile tissue culture

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1 Flow Laboratories, U.K.

2 Gibco Ltd., Paisley, Scotland, U.K.

tubes for the experiments, the tubes were placed in a roller drum, incubated overnight at 37°C and examined for cloudiness or colour change of the indicator. Any tubes with such changes were discarded. OCM was freshly prepared for each experiment.

Preparation of the tracheal sections

Tracheal sections were prepared under aseptic conditions as described by Cherry and Taylor-Robinson (1970a). Chick embryos of 19 day of age and duck embryos of 25 days were used.

The blunt end of the egg was swabbed with Merthiolate[1] solution, and then opened for removal of the embryo. The embryo was decapitated and the trachea was excised between the larynx and syrinx and freed from any connective tissue. It was then cut into transverse sections of approximately 0.6 mm using a McIlwain Tissue Chopping machine[2] and the sections placed in a sterile Petri dish containing OCM. Each chick embryo trachea yielded more than 15 rings while a duck trachea yielded at least 30 rings. Each ring was then placed in a sterile disposable screw-top plastic tissue culture tube[3] containing 1 ml of OCM and allowed to adhere to the side about 20 mm from the bottom. The tubes were then placed in a roller drum (10 rev/hr) and incubated at 37°C. At the daily examination of the rings during each experiment, they were detached from the side of the tube and shaken vigorously to avoid blockage of the lumen by debris.

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1 Eli Lilly and Co. Ltd., Basingstoke, U.K.
2 Mickle Laboratory Engineering Co. Ltd., U.K.
3 Nunclon InterMed, DenmarK.

Selection and assessment of tracheal sections

Tracheal sections were selected after overnight incubation by examination under an inverted microscope[1] using x10 magnification so that the whole ciliated lumen was in the field. Two criteria were used for selecting the sections: the extent of ciliary activity and the vigour of ciliary movement (Cherry and Taylor-Robinson, 1970a). The extent of the ciliary activity in each ring was estimated and the ciliary vigour was arbitrarily graded on scale from 0 to 4. Tracheal sections that possessed 100% ciliary activity with vigour graded as 4 were selected for use.

After infection as described in the subsequent experiments, tracheal rings were examined daily and scored for ciliary activity. The time taken in days for 50% of an experimental group of organ cultures to exhibit total absence of the ciliary activity was also recorded.

Mycoplasma inocula

Stock cultures of strains 4229 and B2/85 (3 times cloned) were used to grow up sufficient inocula for these experiments and stored at -60°C as 1 ml aliquots. A sample of each was thawed and viable counts (2.8) carried out to determine the titre of each culture. The results are shown in Table 7:1 along with the passage levels.

M.gallisepticum S6LP and S6HP were used directly from the frozen stocks referred to in 2.7. The viable counts and passage levels are also shown in Table 7:1.

For the experiments, a thawed aliquot was diluted to provide the required inoculum dose by making serial tenfold dilutions using MB until the final dilution, which was made in OCM. For all experiments 0.1 ml of the suspension of mycoplasmas in OCM

1 E.Leitz (Instruments) Ltd., London, U.K.

Table 7:1 Details of mycoplasma strains used in organ culture experiments

Strain	CFU/ml	Passage level
4229	6.4×10^7	12[1]
B2/85	2.9×10^7	12
S6LP	7.8×10^8	4
S6HP	1.3×10^9	105

1. No. of *in vitro* passages in our laboratory

was inoculated into tubes containing a tracheal ring in 1 ml of OCM so that there was only one part in 100 of MB in each tube.

Sampling of infected and control TOCs

Viable counts were carried out on the inocula and then at 2 day intervals during the experiments on pooled supernatant fluid prepared by taking 50 µl from the first 5 infected tubes in each set of 20. The identity of the mycoplasmas was confirmed by IFA (6.2.2) on a colonies of each strain selected from the viable counts.

The uninoculated control group in each experiment was checked for mycoplasma contamination by pooling 50 µl volumes of supernatant from five tubes, inoculating onto MA and examining for mycoplasma growth as described in 2.5. Mycoplasmas were not recovered from any samples in the following series of experiments.

7.1.3 Experiments and results

Experiment 1. Growth of strains 4229 and B2/85 in OCM (a) with and (b) without the addition of 5% calf serum

The growth characteristics of strains 4229 and B2/85 were first examined over 6 days in OCM without tracheal sections but with or without the addition of calf serum. The experiments were carried out using a low dose (approximately 10^3 CFU) and a higher dose (approximately 10^6 CFU) of inoculum, and *M.gallisepticum* S6LP and S6HP were included for comparison. Titrations of the organisms in MB were run in parallel using the same inocula.

All cultures were set up under the same conditions as organ cultures , i.e. in 1 ml volumes in disposable plastic screw-capped tubes and incubated on a roller drum.

Five tubes were used per group and a further group of five containing uninoculated OCM was included as a medium control. Another group of five tubes containing uninoculated MB was included as a control for the MB cultures. Viable counts were performed on the inocula and then on pooled 50 µl samples taken at 2 day intervals from the inoculated groups. Change in colour of the pH indicator was also recorded.

(a) OCM with calf serum

The results using the lower dose of inoculum are shown in the Figs 7:1 and 7:2. All four strains showed a decline in viability in OCM with serum and the phenol red indicator remained unchanged (Fig7:1). *M.gallisepticum* S6LP showed the most rapid decline but none of the strains were viable by day 4. In contrast, all four strains showed multiplication accompanied by an acid colour change in MB (Figure7:2). Viability declined rapidly with strains 4229 and B2/85 after 2 days but was maintained above 10^6 with *M.gallisepticum.*

Using the higher dose of inoculum (10^6 CFU) did not radically alter the results. None of the 4 strains grew in OCM (Figs 7:3), as indicated by the decline in viability and lack of colour change in the indicator. In MB (Figure7:4) the S6 strains showed an increase in titre, while strains 4229 and B2/85 showed a decline in titre although the yellow colour change indicated that growth, followed by rapid death, must have occurred.

(b) OCM without calf serum

There was loss of viability of all 4 strains in OCM without serum (Figure7:5 and 7:7) and the phenol red indicator remained unchanged, whereas the same inocula presented to MB resulted in obvious growth of strains S6LP and S6HP (Figure7:6 and 7:8). Again a decrease in titre was recorded for strains 4229 and B2/85 but acid production indicated that they had grown in the MB.

Fig. 7:1 Experiment 1a. Growth in OCM with serum

(low inoculum dose)

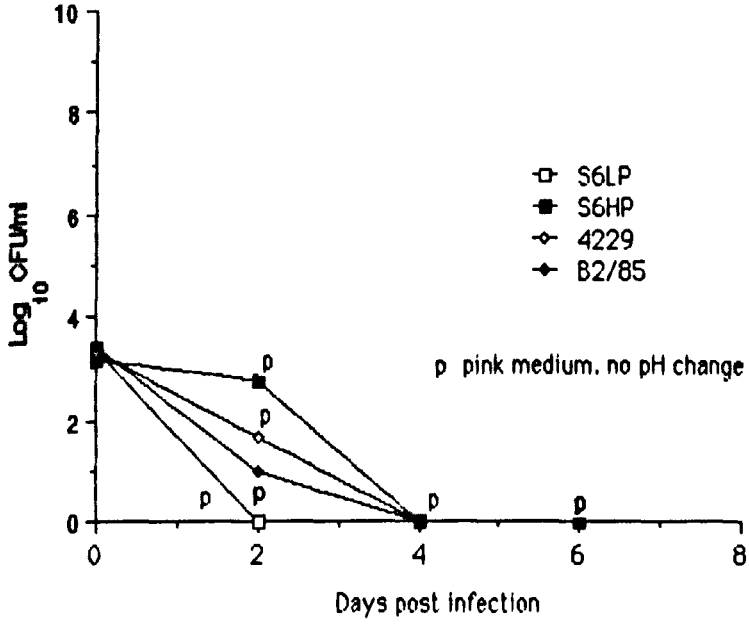


Fig. 7:2 Experiment 1a. Growth in MB

(Control for inoculum used above)

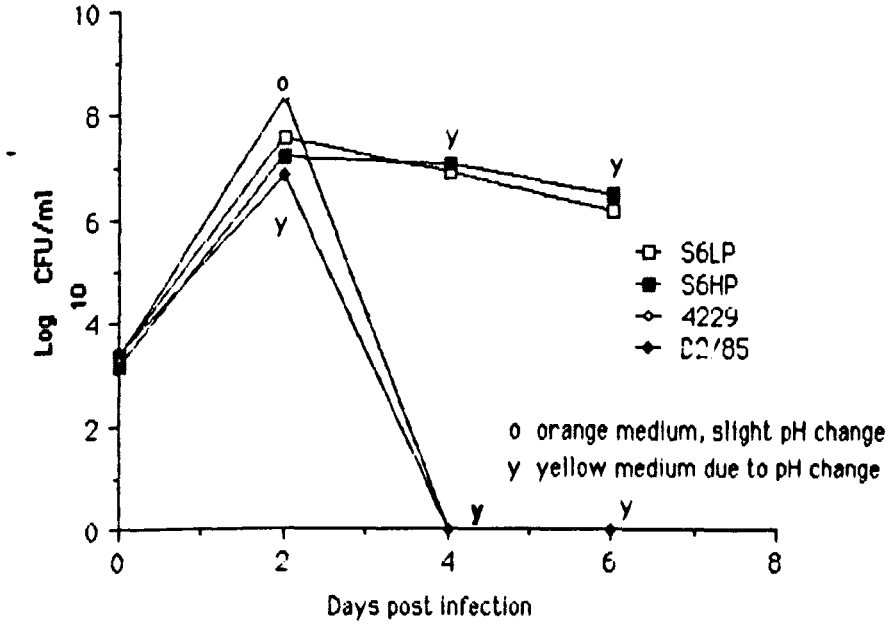


Fig. 7:3 Experiment 1a. Growth in OCM with serum (high inoculum dose)

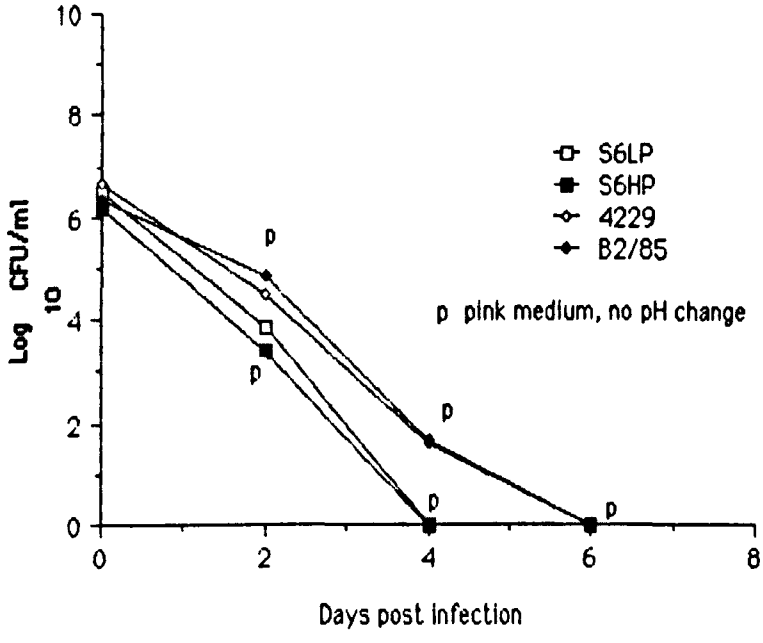


Fig. 7:4 Experiment 1a. Growth in MB (Control for inoculum used above)

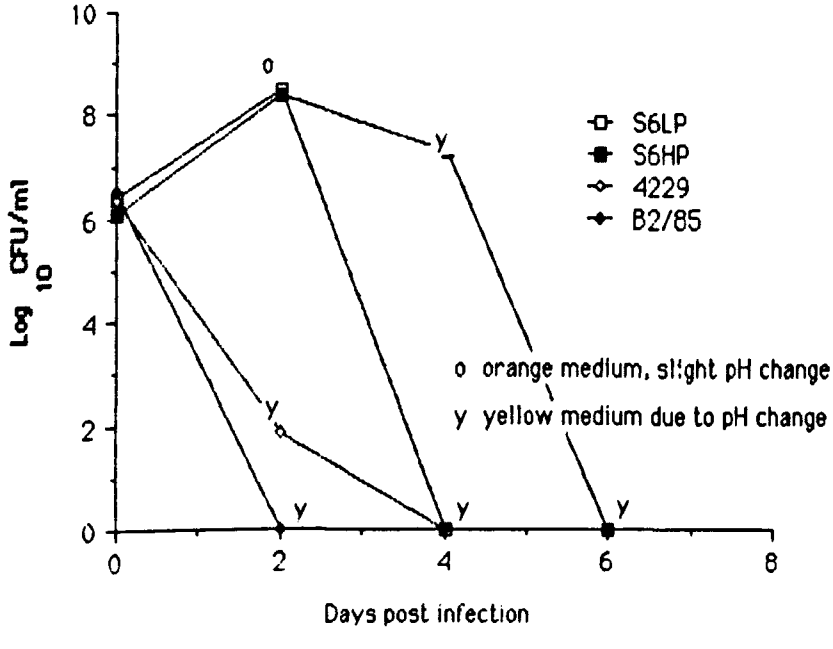


Fig. 7:5 Experiment 1b. Growth in OCM without serum

(low inoculum dose)

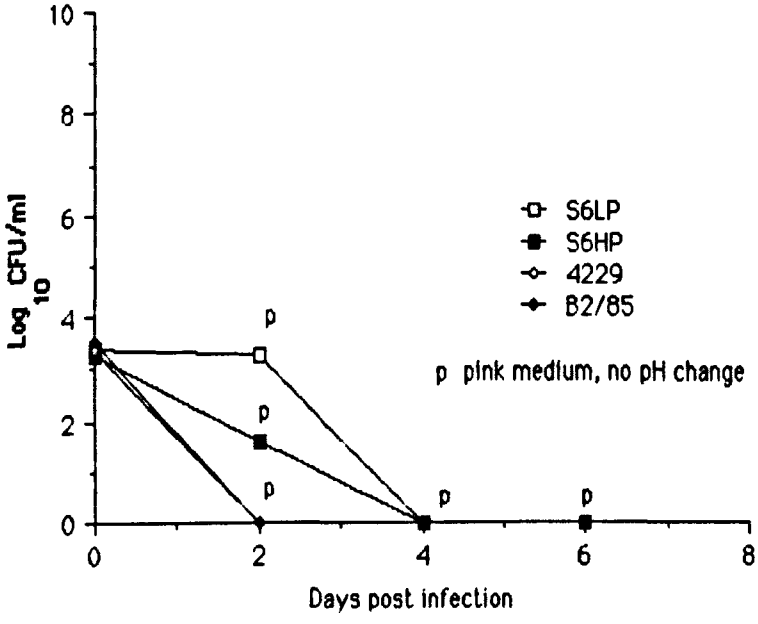


Fig. 7:6 Experiment 1b. Growth in MB

(Control for inoculum used above)

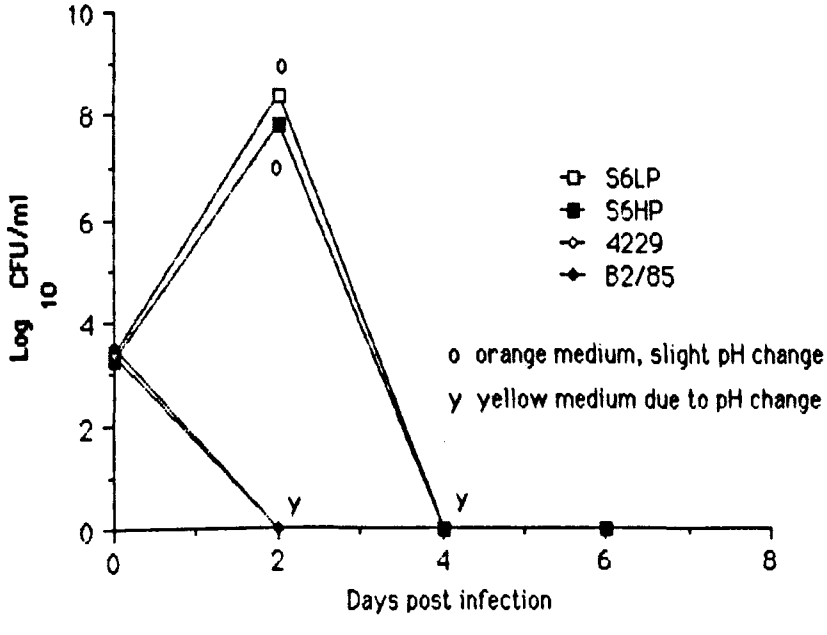


Fig. 7:7 Experiment 1b. Growth in OCM without serum
(high inoculum dose)

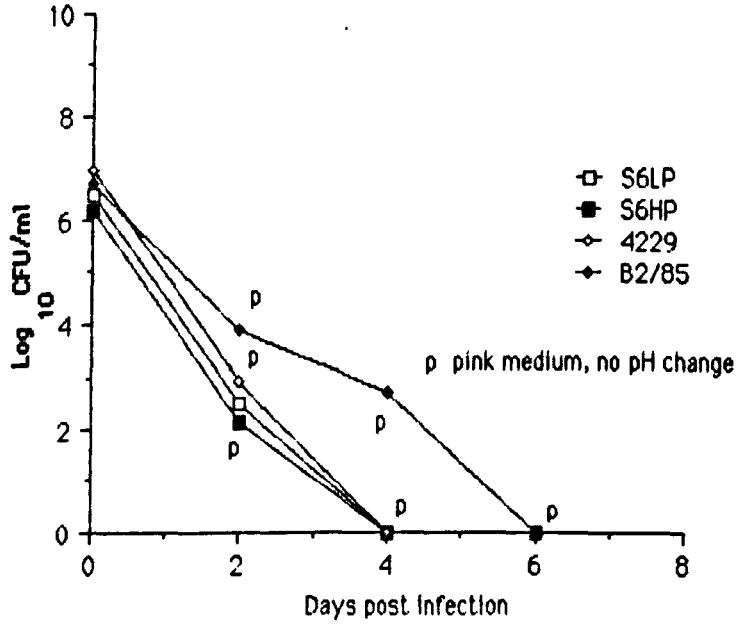
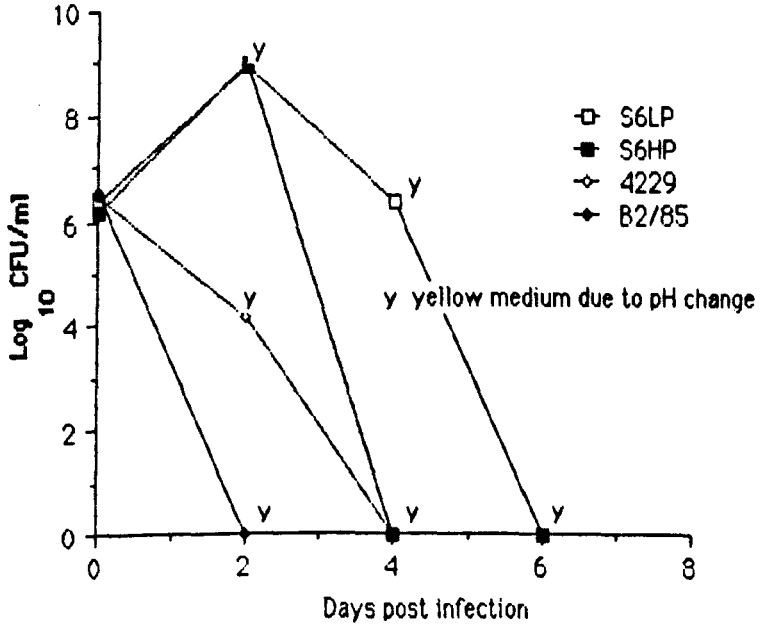


Fig. 7:8 Experiment 1b. Growth in MB
(Control for inoculum used above)



Experiment 2. The growth and ciliostatic effect of strains 4229 and B2/85 in chick embryo TOCs in OCM (a) with and (b) without calf serum

Experiment 1 indicated that OCM, with or without calf serum, did not support the growth of strains 4229 or B2/85 and the next experiments were carried out to investigate the effects of the mycoplasmas on chick TOCs by using the same conditions as above but with the addition of a tracheal ring to each tube. Three different dose levels (intended to be 10^3 ('low'), 10^5 ('medium') and 10^6 ('high') CFU) were used, and as before, *M.gallisepticum* S6LP and S6HP were included for comparison. A set of 20 replicate TOCs was used for each strain and each dose level. A further control group of 20 tracheal rings was inoculated with OCM plus or minus calf serum, as appropriate.

(a) OCM with calf serum

The results using the inoculum of 10^3 CFU are given in Table 7:2 and Figs 7:9 and 7:10. Ciliostasis was observed in all infected groups but not in the control group (Table 7:2 and Figure7:9). The ciliostasis, which was accompanied by acid production, was more rapid with strain S6LP than with S6HP, which in turn was more rapid than with two untyped strains. There was growth of all 4 strains in the organ cultures as indicated by an increase in the titre (Figure7:10). The S6 strains showed slightly higher peak titres than the other two strains. The identity of the four strains was confirmed by IFA.

Results using intended inoculum doses of 10^5 and 10^6 CFU are shown in Table 7:2 and Figs 7:11-7:14. S6LP again exhibited more rapid ciliostasis than S6HP which was generally more rapid than that of the two untyped strains. When inoculated at the highest dose, there was no evidence of multiplication of strains 4229 and B2/85 and only a small increase in titre was seen in the S6 strains (Figure 7:14) while at the slightly lower inoculum level there was evidence of growth (Figure 7:12).

Table 7:2 Summary of the ciliostatic effect of strains 4229, B2/85, S6LP and S6HP on chick or duck embryo TOCs

		Time taken for 50% ciliary inhibition (days)				
Expt. no.	Dose	Control	4229	B2/85	S6LP	S6HP
2a.						
Chick[1] + serum[2]	low	>13	8	9	6	7
Chick + serum	medium	>14	7.5	11	6.5	7.5
Chick + serum	high	>14	10.5	9	4	6
2b						
Chick - serum	low	>14	10	11	8.5	7.5
Chick - serum	high	>14	8.5	5	8	7.5
3a						
Duck + serum	low	>13	7	7.5	4	5
Duck + serum	high	>8	6	6	3.5	4.5
3b						
Duck - serum	low	>15	11	10	5	8

1. Origin of organ culture (chick or duck embryo)
2. Presence or absence of calf serum

Fig. 7:9 Experiment 2a. The ciliostatic effect of strains 4229, B2/85, *M.gallisepticum* S6LP and S6HP on chick embryo TOCs
(OCM with serum; low inoculum dose)

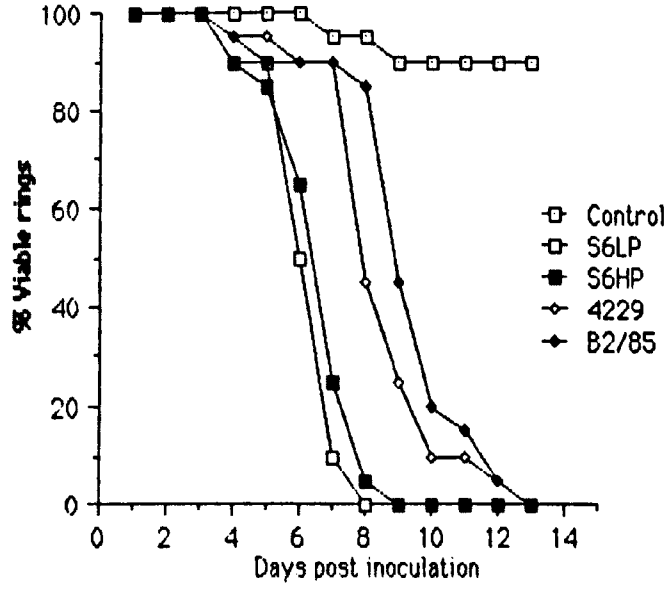


Fig. 7:10 Experiment 2a. Growth of the mycoplasma strains in chick embryo TOCs
(OCM with serum; low inoculum dose)

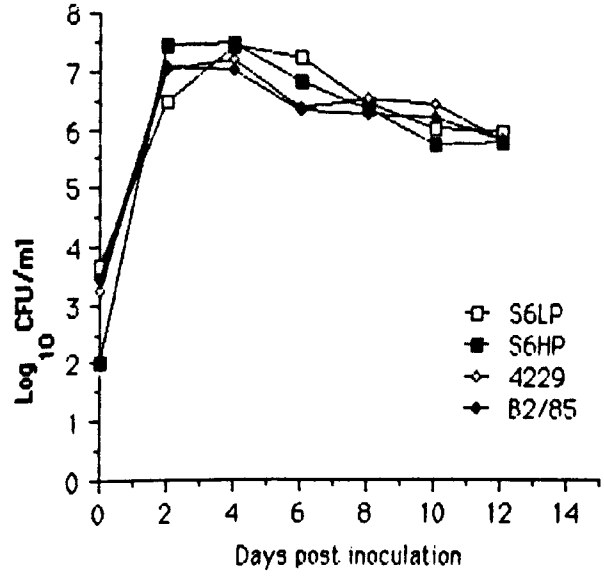


Fig. 7:11 Experiment 2a. The ciliostatic effect of strains 4229, B2/85, *M.gallisepticum* S6LP and S6HP on chick embryo TOCs
(OCM with serum; medium inoculum dose)

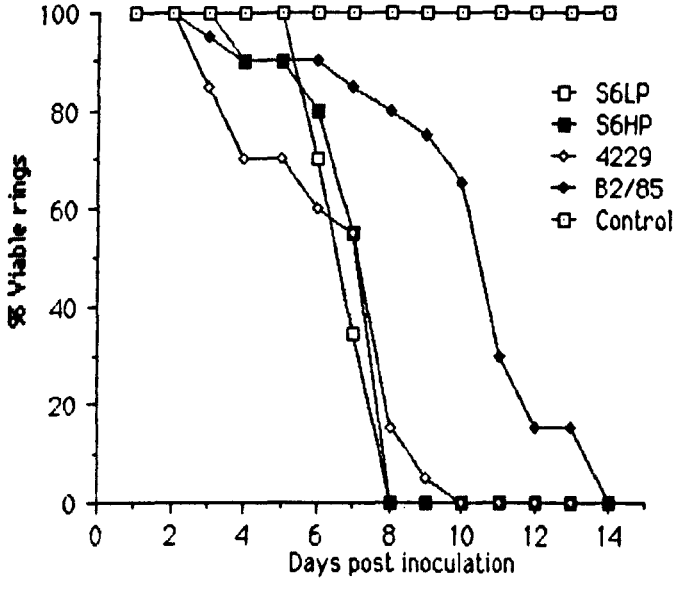


Fig. 7:12 Experiment 2a. Growth of the mycoplasma strains in chick embryo TOCs
(OCM with serum; medium inoculum dose)

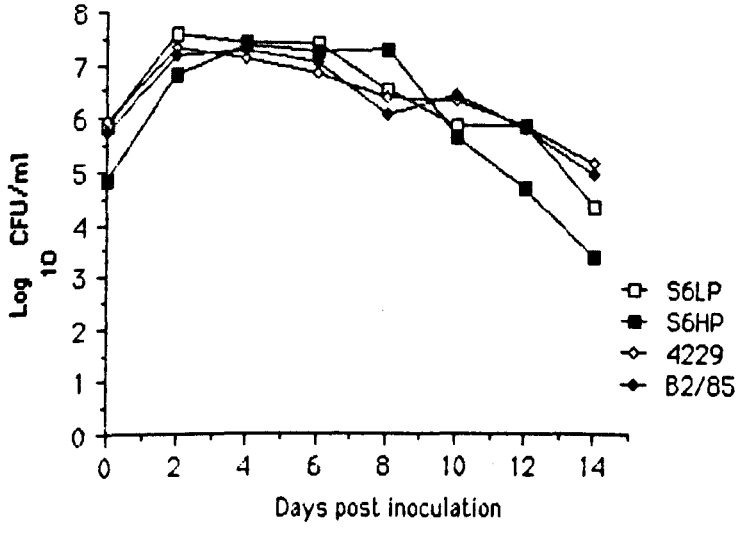


Fig. 7:13 Experiment 2a. The ciliostatic effect of strains 4229, B2/85, *M.gallisepticum* S6LP and S6HP on chick embryo TOCs
(OCM with serum; high inoculum dose)

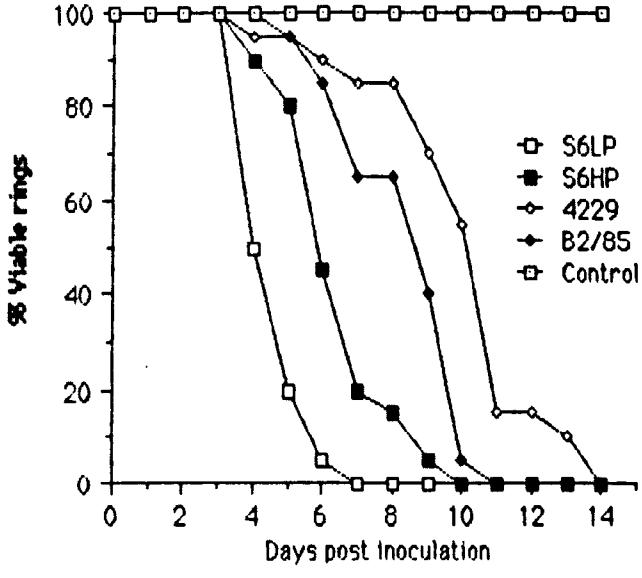
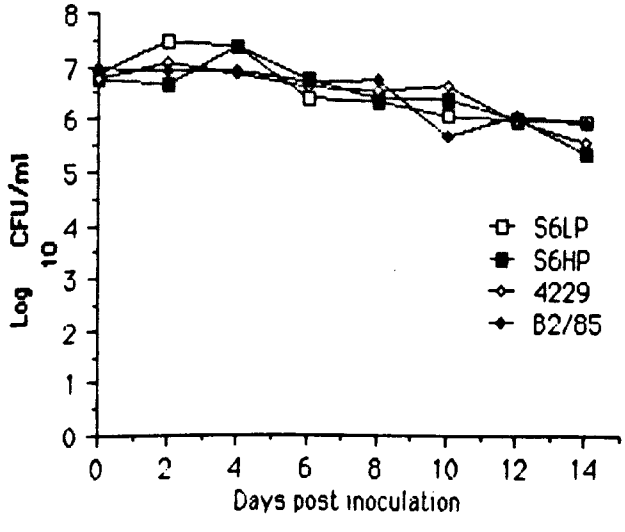


Fig. 7:14 Experiment 2a. Growth of the mycoplasma strains in chick embryo TOCs
(OCM with serum; high inoculum dose)



(b) OCM without calf serum

Two groups of chick embryo TOCs in OCM without calf serum were infected as above using intended inoculum titres of 10^3 and 10^6 CFU. The results are presented in Table 7:2 and Figs 7:15, 7:16, 7:17 and 7:18. In both experiments, ciliostasis accompanied by acid production was exhibited by the organ cultures infected with all mycoplasma strains. With the lower inoculum dose the effects were more rapid with *M.gallisepticum* strain S6LP and HP than the two untyped strains, with S6HP strain giving slightly earlier time of 50% reduction in ciliostasis. In the experiment with larger inoculum size strain B2/85 caused the earliest 50% reduction and it also showed the most active growth.

Experiment 3. The growth and ciliostatic effect of strains 4229 and B2/85 in duck embryo TOCs in OCM (a) with and (b) without calf serum

(a) OCM with calf serum

Two inoculum titres (approximately 10^3 and 10^6 CFU) were used and the experimental procedures were exactly as described for the chick TOCs.

The results are given in Table 7:2 and Figs 7:19, 7:20, 7:21 and 7:22. The control groups in this experiment did not maintain ciliary vigour for as long as the chick TOCs but were satisfactory for comparison with the infected groups. Ciliostasis accompanied by acid production was shown by all 4 strains at both dose levels. It was slightly more rapid with *M.gallisepticum* strain S6LP than S6HP and both these strains caused earlier ciliostasis than the other two strains (Table 7:2 and Figs 7:19 and 7:21). There was evidence of growth of all 4 strains following inoculation at the lower dose, but there appeared to be only a slight increase in viability with the higher dose.

Fig. 7:15 Experiment 2b. The ciliostatic effect of strains 4229, B2/85, *M.gallisepticum* S6LP and S6HP on chick embryo TOCs
(OCM without serum; low inoculum dose)

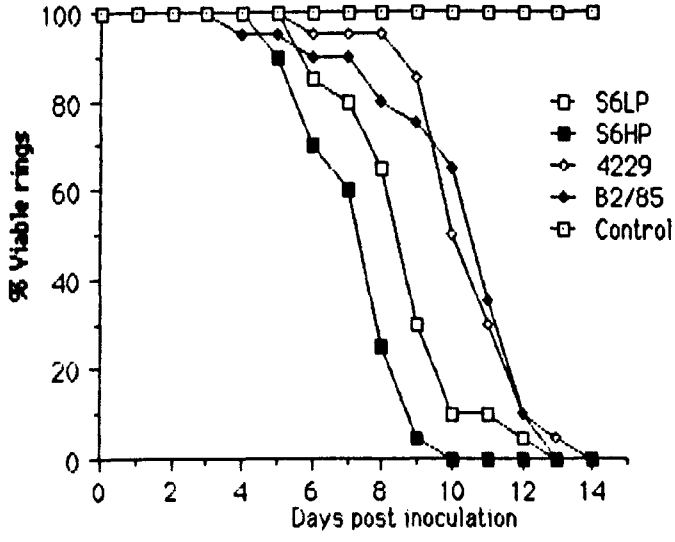


Fig. 7:16 Experiment 2b. Growth of the mycoplasma strains in chick embryo TOCs
(OCM without serum; low inoculum dose)

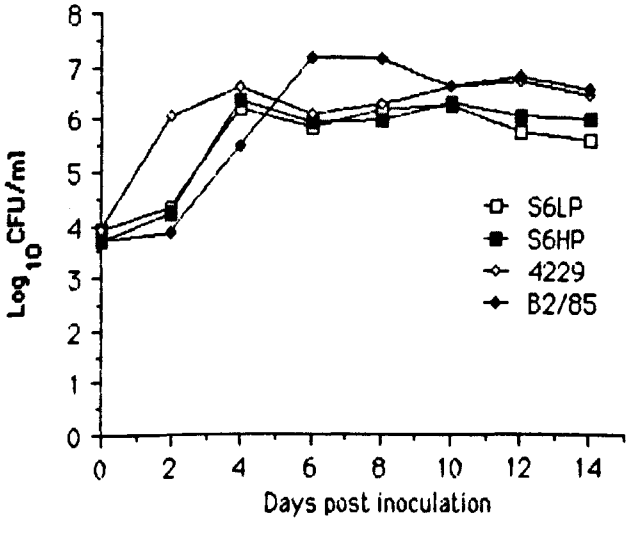


Fig. 7:17 Experiment 2b. The ciliostatic effect of strains 4229, B2/85, *M.gallisepticum* S6LP and S6HP on chick embryo TOCs
(OCM without serum; high inoculum dose)

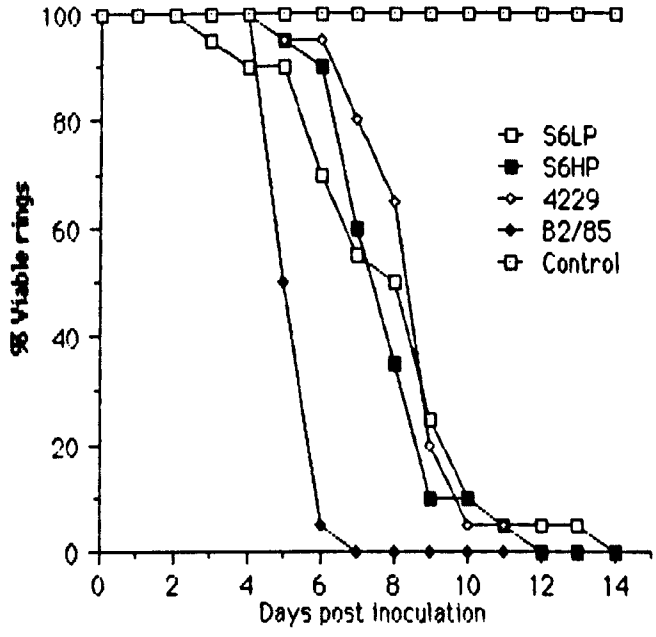


Fig. 7:18 Experiment 2b. Growth of the mycoplasma strains in chick embryo TOCs
(OCM without serum; high inoculum dose)

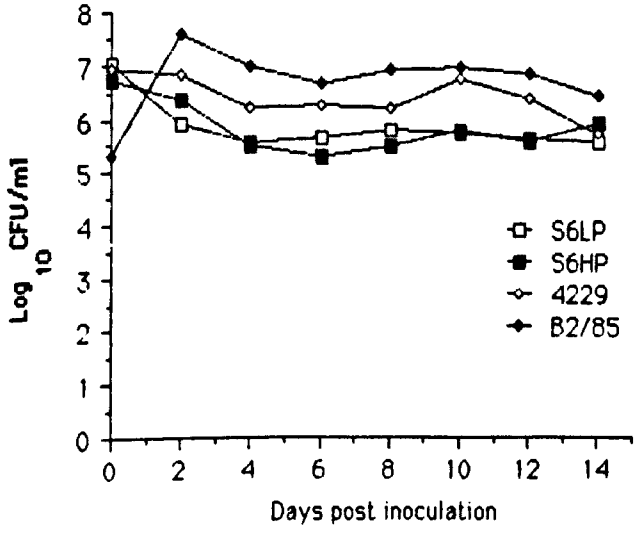


Fig. 7:19 Experiment 3a. The ciliostatic effect of strains 4229, B2/85, *M.gallisepticum* S6LP and S6HP on duck embryo TOCs
(OCM with serum; low inoculum dose)

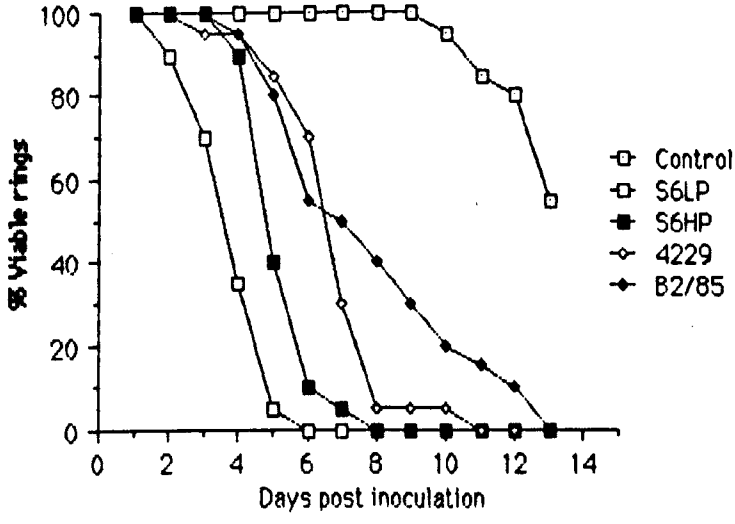


Fig. 7:20 Experiment 3a. Growth of the mycoplasma strains in duck embryo TOCs
(OCM with serum; low inoculum dose)

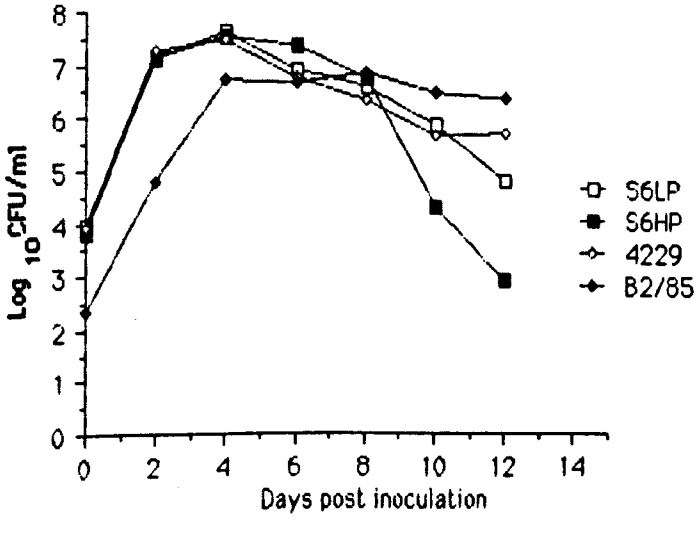


Fig. 7:21 Experiment 3a. The ciliostatic effect of strains 4229, B2/85, *M.gallisepticum* S6LP and S6HP on duck embryo TOCs
(OCM with serum; high inoculum dose)

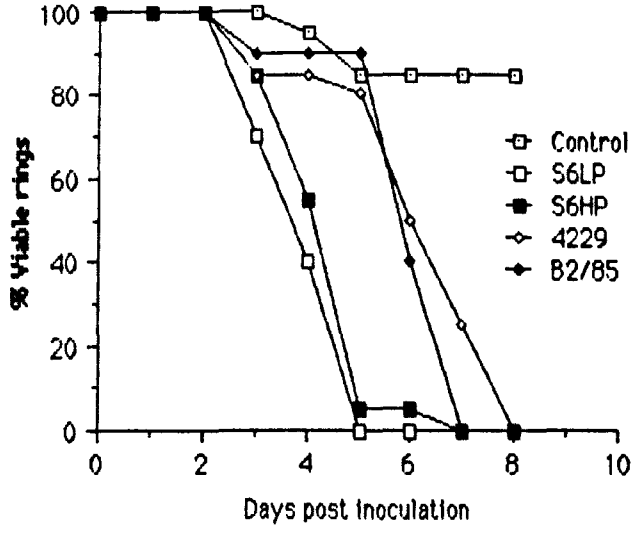
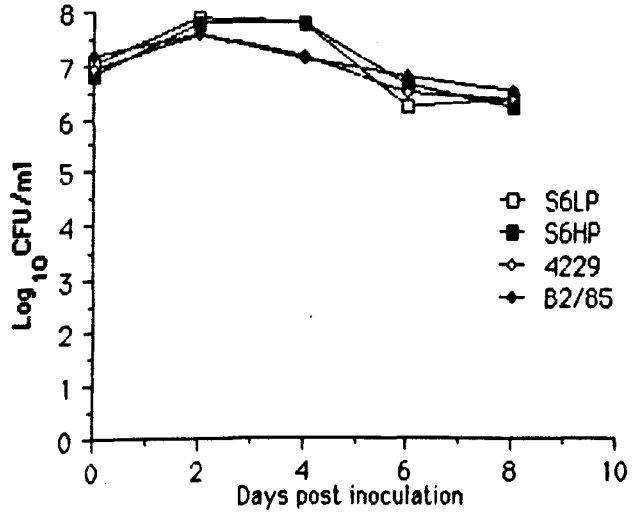


Fig. 7:22 Experiment 3a. Growth of the mycoplasma strains in duck embryo TOCs
(OCM with serum; high inoculum dose)



(b) OCM without calf serum

Since the titre of the inocula did not appear to have a significant effect on the outcome of these experiments, only the lower dose was used for the duck TOCs in OCM without serum.

The results are shown in Table 7:2 and Figs 7:23 and 7:24. Ciliostasis accompanied by acid production was shown by all infected groups and again it was more rapid in the cultures infected with *M.gallisepticum* strain S6LP than S6HP which in turn was slightly more rapid than the two untyped strains. All the strains grew well in the duck organ culture without serum (Figure7:24).

7.1.4 Discussion

The fertile chicken eggs used throughout the experiments to prepare TOCs were from the Department's own SPF flock but the duck eggs were supplied by a commercial company. There was no evidence of mycoplasma infection from either source.

The chicken eggs were incubated for 19 days and the duck eggs for 25 days because the later stage of incubation is considered to be more suitable than earlier stages for preparation tracheal sections since the ciliary activity is stronger and less susceptible to traumatic shock.

Tracheal sections prepared from these eggs were satisfactory since they remained viable for at least 10-14 days without a change of medium . In each experiment they were randomised from different parts of the trachea and from different embryos so that variation in host tissue was unlikely to affect the results within individual experiments. Cherry and Taylor-Robinson (1973) obtained different results when they used two different series of experiments in chick embryo TOCs to study the

Fig. 7:23 Experiment 3b. The ciliostatic effect of strains 4229, B2/85, *M.gallisepticum* S6LP and S6HP on duck embryo TOCs
(OCM without serum; low inoculum dose)

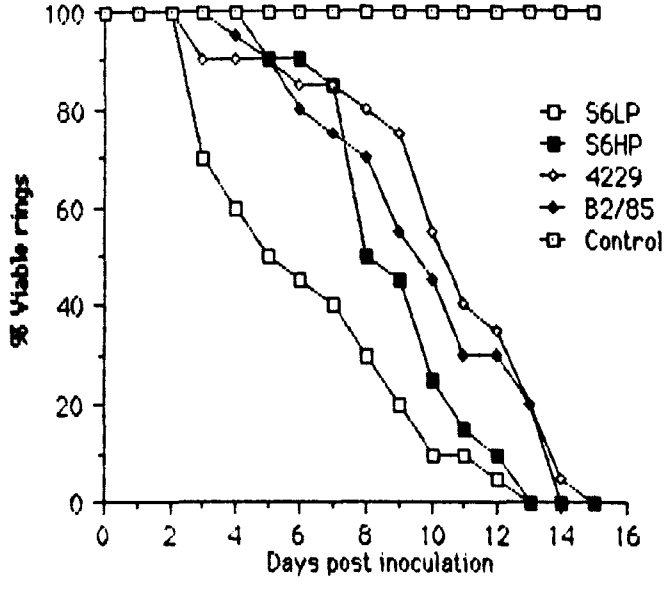
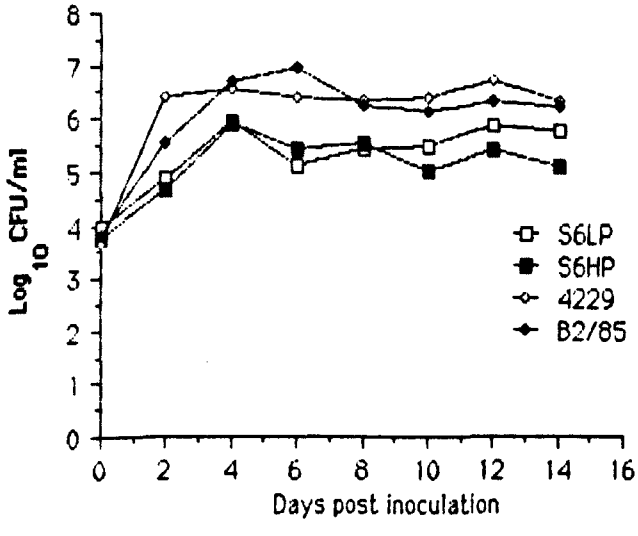


Fig. 7:24 Experiment 3b. Growth of the mycoplasma strains in duck embryo TOCs
(OCM without serum; low inoculum dose)



growth of *M.pneumoniae* strains. Ciliostasis occurred only in one series and it was considered that this was due to the better quality of the rings.

The scoring system described by Cherry and Taylor-Robinson (1970a) used two criteria, extent and vigour of ciliary activity, but they only assessed small numbers of tracheal sections (five per group). Engelhardt and Gabridge, (1977) presented the measurement of relative ciliary activity of hamster TOC sections as the percentage of the epithelial layer that remained intact, multiplied by the relative vigour of ciliary beating graded on a scale of 0 to 3. In our experiments results were based simply on presence or absence of ciliary activity but with larger numbers of replicate samples.

The medium used was based upon that described by Cherry and Taylor-Robinson (1970a) which in their case maintained the ciliary activity of chick embryo TOCs for at least 10-15 days without changing the medium.

The mycoplasma inocula were prepared by making ten-fold dilutions in MB, except for the final dilution which was always made up in OCM, with or without serum as appropriate. The final dilution in OCM was to avoid the inhibitory effect of the thallium acetate in MB on the organ cultures (McClenaghan, 1982) and also to avoid addition of significant amounts swine serum and of glucose which might give rise to acid production. Other workers have used a variety of media for inoculation of TOCs. Power and Jordan (1976) used inocula in MB containing thallium acetate for their chick embryoTOC experiments, although they suggested prior screening of batches of broth was necessary to select those that caused little or no ciliostasis. Those batches which proved suitable might be those in which the thallium acetate had precipitated out. Other workers who have used mycoplasma broth to prepare inocula have omitted the thallium acetate (Cherry and Taylor-Robinson, 1970a; Abu-Zahr and Butler, 1976).

The type of medium used for inoculation and for culturing of tracheal explants must also take into consideration the presence or absence of serum. Two schools of investigators exist according to whether they use medium with serum (permissive medium, i.e. that capable to supporting mycoplasma metabolism and replication) or without serum (non-permissive medium; i.e. which does not by itself support mycoplasma growth).

The first group includes Collier and co-workers (1969) who used mycoplasma medium containing 20% horse serum to propagate the mycoplasmas and to support infected human embryo TOCs. Gabridge and Barden Stahl (1978) used mycoplasma medium supplemented with 10% serum to grow the inoculum and then Minimal Essential Medium (MEM) supplemented with 10% serum to support hamster TOCs infected with *M.pneumoniae* .

In the second group, Butler (1969) used human foetal TOC cultured in MEM without added serum, and Cherry and Taylor-Robinson (1970a) tested mycoplasma strains grown in MB and inoculated into chick embryo TOCs cultured in MEM without added serum. They suggested that although organ culture medium did not itself support growth of the tested mycoplasmas it provided conditions that were near to those found in the intact animal.

In our experiments it was decided to study the effect and the growth of strains 4229 and B2/85, in comparison with *M.gallisepticum* strains S6LP and S6HP, on chick embryo and duck TOCs cultured in OCM, and to investigate the effect of presence or absence of serum in the medium.

In the initial experiments on growth in OCM with or without addition of serum but without tracheal rings, there was no evidence of multiplication of these strains, or of *M.gallisepticum* S6LP and S6HP, and this was not influenced by the initial dose of

inoculum. This lack of growth was consistent in all four titration series that were performed. These results, when compared with those of the later experiments, demonstrated that multiplication of the mycoplasmas was dependent upon the presence of TOCs. Since the addition of 5% calf serum to the OCM did not encourage growth of any of the strains in the absence of tracheal rings, it was considered that OCM, with or without serum was a non-permissive medium whereas OCM containing a tracheal section, with or without serum, supported growth of all four strains, and was considered to be permissive.

Absence of growth in OCM was in agreement with the observations of other workers. Abu-Zhar and Butler (1976) found that neither *M.gallisepticum* nor *M.gallinarum* were able to multiply in organ culture medium alone but they both grew well in medium containing chick embryo tracheal explants. Hirano *et al.* (1978) found that *M.gallisepticum* S6 showed little or no growth in organ culture medium without tracheal rings but that growth of this mycoplasma and *M.synoviae* occurred in organ cultures with rings, independent of the presence of porcine serum incorporated in the organ culture medium. In another study Takagi and Arakawa (1980) observed that *M.gallisepticum* grew only in presence of tracheal rings in organ culture medium, and that growth was independent of the presence of serum. Similarly, growth of *M.mycoides* subsp. *capri* in chick embryo TOCs was closely associated with the tracheal tissue (Cherry and Taylor-Robinson, 1970b). Reed (1972) also observed that bovine TOCs supported growth of *M.hyorhinis* in contrast to the same medium without tissues.

Requirement for tracheal epithelial cells for growth of *M.pneumoniae* has been demonstrated by Chen and Krause (1988) who found that viable organisms increased more than 30-fold when cultured in the presence of hamster tracheal epithelial cells but less than one-fold without cells.

In our experiments with both chick and duck embryo TOCs all four mycoplasma strains grew, and all four caused ciliostasis, accompanied by acid production, independent of the dose inoculated and the presence or absence of serum. In the presence of serum peak viable counts tended to occur earlier than in its absence although when inoculated at a dose of 10^7 CFU they showed little or no increase. The rapidity of ciliostasis varied from one experiment to another but, as with the viable counts, it tended to occur earlier when serum was present. It was usually more rapid with *M.gallisepticum* S6LP and S6HP than with strains 4229 and B2/85 although there appeared to be an anomaly with strain B2/85 in the chick embryo TOC experiment without added serum and high inoculum because it exhibited the most rapid ciliostasis. These results might be explained by the apparent absence of the growth of the other strains.

Although ciliostasis was generally more rapid with strain S6LP than with S6HP there was little difference between them in the number of days taken to reach 50% ciliostasis. Similarly, the ciliostasis was usually more rapid with strain 4229 than B2/85 but little difference occurred in the timing of 50% ciliostasis.

The inhibition of ciliary activity by *M.gallisepticum* strains in chick embryo TOCs is in agreement with the findings of other workers. Cherry and Taylor-Robinson (1970a) found that 7 out of 7 strains of *M.gallisepticum* caused ciliostasis using OCM without addition of serum. Butler and Ellaway (1971) showed that several strains of *M.gallisepticum*, including strain S6, had a ciliostatic effect on chick embryo TOCs using OCM without addition of serum. In another study, Abu-Zahr and Butler (1976), also using OCM without addition of the serum, observed that *M.gallisepticum* S6 grew well in chick embryo TOCs, inducing rapid ciliostasis,.

Ciliostasis by *M.dispar* in bovine fetal TOCs had been found to be dependent on the presence of foetal calf serum in the maintenance medium (Howard and Thomas,

1974). These workers also explained that the ciliostasis was due to better growth in the tracheal rings maintained in basal medium containing the calf serum, and was related to the number of the viable organisms.

To the best of our knowledge, duck TOCs have not been used before to study avian mycoplasmas. Butler and Ellaway, (1971) found that the capacity of TOCs of different hosts to support the growth of mycoplasmas might vary, since they found that growth of different mycoplasma species varied in human or chicken embryo organ cultures. For example: *M.salivarium* and *M.orale* grew in human but not chicken embryo TOCs while *M.gallisepticum* and *M.mycoides* subsp.*capri* grew in both types of TOC. However our experiments have demonstrated that strain 4229 and B2/85, and also the S6 strain of *M.gallisepticum* grow equally well in both chicken and duck embryo TOCs.

The difference in rapidity of ciliostasis that we observed between the S6LP and S6HP strains of *M.gallisepticum* in the chick embryo TOC experiments was in agreement with other workers. Power and Jordan (1976) found that strain S6LP suppressed ciliary activity more rapidly than two other strains of *M.gallisepticum* including S6HP. There was a slight difference between our studies and theirs in the times required for 50% ciliostasis, ours being a little longer. This was possibly due to the difference in the presentation of the inoculum, theirs being in MB with thallium acetate.

It has been found that some mycoplasma species do not inhibit ciliary activity in spite of their multiplication in organ cultures (Cherry and Taylor-Robinson, 1970a; 1973), indicating that ciliostasis is not necessarily a result of multiplication of organisms but more likely to be influenced by their pathogenicity.

In our experiments there did not appear to be any difference between chicken and duck embyo TOCs in the relationship between the number of viable organisms and ciliostasis. In both the maximum ciliostasis did not correspond with the maximum number of viable organisms, but instead it was reached when the growth curves were already in decline. This was seen in all experiments. However, the viable counts represent the number of colony forming units in the supernatant of the TOCs detached from the tracheal organ surface, and exclude those on the tracheal luminal surface. Thus the viable counts are only an indirect reflection of the multiplication of the organisms on the tracheal surface.

The ciliostasis of strains 4229 and B2/85 or of the S6 strains was unrelated to the inoculum dose since the use of the different inoculum titres resulted in little difference in the timing of 50% ciliostasis. Lack of a relationship between the dose of *M.gallisepticum* and ciliostasis was observed by Cherry and Taylor-Robinson (1971). In contrast, Levisohn *et al.* (1986) found that ciliostasis produced by certain strains of *M.gallisepticum* in chicken TOCs was proportional to the size of inoculum. A difference between a low and a high passage strain of S6 was seen at a low dose of inoculum (5 to 50 CFU) but it was partly obscured at a higher dose ($5x10^5$ CFU). They suggested that the lack of relationship between dose and ciliostasis that had been seen in the earlier studies of Cherry and Taylor-Robinson (1971) was due to the use of a high dose level.

In contrast to their findings with *M.gallisepticum*, Cherry and Taylor-Robinson (1970b) observed that the rapidity of ciliostasis in chick embryo TOCs infected with *M.mycoides* subsp. *capri* was related directly to the number of organisms inoculated. Similarly, Collier *et al.* (1969) observed that the ciliostatic effect of *M.pneumoniae* in hamster TOCs was related to the dose of inoculum and a similar relationship was observed by Butler and Ellaway (1971) with *M.mycoides* subsp. *capri* in human embryo TOCs. Furthermore Stadtlander and Kirchhoff (1988) showed that the

inoculum dose of *M.mobile* was related strongly to the severity of ciliostasis occurring in rat and piglet TOCs.

The lack of relationship between inoculum dose and ciliostasis in our studies might be because the factors controlling rate of damage were more tissue-cell dependent than dependent on the organisms, and the cells might have specific receptor sites for toxin (Cherry and Taylor-Robinson, 1973). If the number of these sites were few there would be no observed difference in the rapidity of ciliostasis with small and large inocula unless the inoculum contained fewer organisms than the number of receptor sites.

The growth of all the strains in chicken and duck embryo TOCs resulted in reduction of pH and was in agreement with other workers. Takagi and Arakawa (1980) observed that growth of *M.gallisepticum* in chicken TOCs resulted in pH reduction.

Our observations on the *in vitro* pathogenicity of strain 4229 in chick and duck embryo TOCs were not quite as expected from the work of Dupiellet (1988) who compared it with *M.gallisepticum* S6 in chick and duck embryo fibroblasts and in embryonated chick and duck eggs. He found that the duck strain was more pathogenic than S6 in the duck cells and embryos while S6 was more pathogenic in the chicken cells and embryos.

It is possible that the use of embryo fibroblast cells has some advantages over TOCs. Gabridge *et al.* (1979) studied the interaction of pathogenic *M.pneumoniae* in human foetal lung fibroblast monolayer cultures and compared results with those in hamster tracheal explant cultures. He showed that the fibroblasts could bind larger numbers of mycoplasmas than tracheal explants. This might be due to an increase in available surface area, and in addition the absence of mucociliary clearance with fibroblasts. The attachment to fibroblasts was 96% specific, and was mediated

through a neuraminidase-sensitive receptor on the host cell, whereas attachment to tracheal explants was only 50% specific. This related to the cut surface of the tracheal explant having large cavernous spaces in the subepithelial region which could physically trap the mycoplasmas. Thus these artificially-formed surfaces and even the outer surface of the trachea can bind the mycoplasmas non-specifically and introduce error into studies on pathogenic mycoplasma attachment. This non-specific attachment was eliminated with fibroblast monolayers. Binding of mycoplasmas to the outer surface of the trachea was also observed in our studies by immunofluorescent staining and is illustrated in the next section of this Chapter.

The fact that strains 4229 and B2/85 inhibited ciliary activity of both chicken and duck TOCs indicated that they may be pathogenic *in vivo*. Correlation between the effect of *M.gallisepticum* strains on tracheal rings and their pathogenicity for chickens has been established (Levisohn *et al.*, 1986), and the changes in the tracheal epithelial surfaces induced by *M.gallisepticum* infection *in vivo* and *in vitro* followed a similar pattern (Dykstra *et al.*, 1985).

The sensitivity of TOCs for the study of pathogenicity of avian mycoplasma is still unclear. Power and Jordan (1976) in their comparison of virulence of three different strains of *M.gallisepticum* using *in vitro* and *in vivo* methods, found that chick embryo TOCs were less sensitive than the chick embryo for assessing virulence. Hirano *et al.* (1978) found that chicken TOCs showed only mild ciliostasis when infected with either of two strains of *M.synoviae* which were more virulent when inoculated into the tracheas of chickens. They suggested that chicken TOCs only exhibit obvious differences in virulence. On the other hand, Levisohn *et al.* (1986) used chicken TOCs to as model for evaluating pathogenicity. They considered the system to be rapid and less subject to environmental variation than live birds, as well as economical with respect to time, space and materials. They suggested that the test

could be used to define relative virulence and could be related to *in vivo* pathogenicity.

The mechanism of pathogenicity of such organisms is not yet understood and further investigations are necessary to elucidate them. Many factors are possibly responsible for the pathogenicity of the mycoplasmas in TOCs. With *M.gallisepticum*, neither cytoadsorption nor peroxide production were responsible for ciliostasis in chick embryo TOCs (Cherry and Taylor-Robinson, 1971). However, it was found that liberation of peroxide was an important factor in the pathogenesis of *M.mycoides* subsp. *capri* infection of chicken embryo TOCs, and its destruction of the ciliated epithelium was prevented by addition of catalase (Cherry and Taylor-Robinson, 1970b). Collier *et al.* (1969) found that the cytopathogenic effect resulting from *M.pneumoniae* infection in hamster TOCs was related directly to the action of the organisms multiplying on or close to the organ culture surface and was not due to the accumulated metabolic byproducts or medium depletion.

Viability and/or metabolic activity of virulent *M.pneumoniae* is required for cell injury to hamster TOCs (Hu, *et al.*,1990). These workers demonstrated inhibition of host cell ribonucleic acid and protein synthesis within 24 hours of infection. The rate of macromolecular synthesis continued to decline as the incubation time increased and was followed by ciliostasis and progressive destruction of the epithelial cells. The effects on macromolecular synthesis could be rescued by shifting the infected organ cultures into medium that was non-permissive to mycoplasma within 24 hours after infection, and under these conditions the mycoplasmas remained attached to the epithelial cells but did not cause further damage. Hu *et al.* suggested that *M.pneumoniae* pathogenesis occurs as a two-step process, the initial step being attachment via the tip structure, allowing virulent organisms to colonize. Then follows the metabolic alteration and other cytopathological effects which appear to result from the multiplication of the organisms accompanied by possible membrane

perturbation, nutritional parasitism, and/or introduction of certain undefined toxic factors to the parasitized cells.

One of the various possible factors in the pathogenicity of strains 4229 and B2/85 in chick and duck embryo TOCs could be the production of acid. Such cytopathic effects have been observed by Barile and Grabowski (1978) in cell culture monolayers and McClenaghan (1982) demonstrated a direct relationship between acidity of the medium and ciliostasis in uninfected turkey TOCs. Lack of relationship between the acid production and ciliostasis was observed by Stadtlander and Kirchhoff (1988) who controlled the pH of rat and pig TOCs infected with *M.mobile* by repeated changing of the medium. They suggested that peroxide produced by the mycoplasma caused the cell damage.

In conclusion, study of the pathogenic effect of strains 4229 and B2/85 has been carried out in TOCs of both chicken and duck embryos. The cytopathic effect, which was demonstrated by the inhibition of ciliary activity of the TOCs, was milder than that of strains S6LP and S6HP of *M.gallisepticum*. The ciliostasis was independent of the presence of 5% calf serum although the addition of serum tended to encourage the growth of the organisms and earlier onset of ciliary inhibition.

These findings in TOCs were supplemented with immunofluorescence and electron microscopy studies, and also with investigations *in ovo* and *in vivo*. These are presented below.

7.2 Immunofluorescence studies on infected chicken and duck tracheal organ cultures

7.2.1 Introduction

Having established that strains 4229 and B2/85 were pathogenic for chick and duck embryo TOCs, this part of the study was undertaken to investigate the location and amount of mycoplasmas in the infected tracheal tissue by immunofluorescent staining.

Examination of the relationship between mycoplasmas and infected tracheal organ cultures using immunofluorescence has been carried out by several workers, and the immunological specificity of the method has allowed the precise location of the organisms to be ascertained (Collier, 1979).

Organ culture techniques using immunofluorescence microscopy were used to examine the pathogenesis of *M.pneumoniae* disease in hamster TOCs and the intimate association of *M.pneumoniae* and the ciliated epithelial cell membrane of hamster TOCs was examined using immunofluorescence and electron microscopy (Collier *et al.*, 1969; 1971). It was found that bright specific fluorescence was limited to the ciliated border of the tracheal lumen (Collier and Baseman, 1973). In another study of the interaction of *M.pneumoniae* with human foetal TOCs by Collier and Clyde (1971) using light, immunofluorescence and electron microscopy immunofluorescence was useful for differentiating cellular debris from the antigen in relation to damaged cells.

Infection of chick embryo TOCs with *M.synoviae* was investigated for the first time using immunofluorescence by Oriel (1976) but results were inconclusive. However Hirano *et al.* (1978) infected chicken TOCs with *M.synoviae* (strain WVU 1853) or

M.gallisepticum (strain S6) and on examination by immunofluorescence found that both organisms appeared to be lining the epithelial cell surface (Hirano *et al.*, 1978).

7.2.2 Materials and Methods

Materials:

Mycoplasma strains

The investigations were performed with strains 4229 and B2/85 and with *M.gallisepticum* S6LP for comparative purposes. The passage history has already been given (Table 7:1).

Tracheal organ cultures

Tracheal organ cultures were prepared from chick and duck embryos as described previously (7.1.2), and OCM without addition of serum was used.

Non-fade mountant

The mountant was described by Johnson and Araujo (1981).

To prepare 100 ml:

PBS (0.1 M) containing 100.0 mg of	
p-phenylenediamine [1]	10.0 ml
Glycerol	90.0 ml

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1 Sigma, Chemical Company, St. Louis, MO, U.S.A.

The final pH was adjusted to 8.0 with carbonate-bicarbonate buffer (0.5 M, pH 9.0), and the mixture stored at -20 ^{o}C in the dark.

Procedure:

Mycoplasma inocula were prepared as described earlier, and three groups of tracheal rings, consisting of 20 rings per group, were inoculated with 0.1 ml of the appropriate strain at a dilution to provide approximately 10^3 CFU per tube. Viable counts were carried out on the inocula.

Duplicate samples of tracheal rings were obtained for immunofluorescence at intervals of 0 days (uninoculated controls), 1, 2, 3 and 5 days after inoculation. The rings were processed by carefully removing them from the tubes into a small aluminium foil 'cup' containing embedding medium (O.C.T. compound[1]). They were immediately snap-frozen by immersion in liquid nitrogen (-196^{o}C). Each sample was placed in a small self-sealing plastic bag and stored at -80^{o}C until sectioning was carried out.

Sections of 4-5 µ were cut with a cryostat[2] and placed on a glass slide. At least 6 serial sections were mounted on the same slide and the slides were prepared in triplicate. They were air dried and fixed in acetone for 10 minutes at room temperature and stained by the indirect immunofluorescence technique as follows:

The section was flooded with diluted specific rabbit antiserum (6.2.1). (A dilution in PBS of 1:160 for antisera and of 1:80 for conjugate was selected after pilot tests using checker board titrations). After incubation for 30 minutes at room temperature in a humid chamber, the slides were rinsed in two changes of PBS for 15 minutes. A

1 Lab-Tek Products, Miles Lab.Inc.,Naperville, Illinois, U.S.A.
2 Bright Instrument Company Ltd., Huntingdon, England

20 µl drop of the diluted conjugate[1] (F-9262; fluorescein labelled goat anti-rabbit globulin-IgG, whole molecule) was added to cover the section and the slides were incubated and washed as before. The section was mounted in a non-fade mountant and examined for specific fluorescence as described in 6.2.2.

All tests included the following controls:

(i) uninfected tracheal ring tissue with test antiserum and conjugate;

(ii) infected tracheal ring tissue with normal rabbit serum and conjugate;

(iii) infected tracheal ring tissue with conjugate.

7.2.3 Results

No fluorescence was observed in the control preparations of either chick or duck TOCs (Figure7:25) but the infected sections exhibited specific fluorescence in the epithelial border at all stages after inoculation.

Strain 4229 demonstrated small fluorescent granules on the epithelial surface of the lumen of both chick and duck TOCs on the first day after inoculation and by two days this had increased into small accumulated masses of brightly fluorescent material (Figure7:26 & 7:27). These increased in amount with time and persisted to the end of the experiment at five days and the fluorescence appeared to extend into the subepithelial layer in some areas (Figure7:28 & 7:29). Similar changes were seen with strain B2/85 except that at 24 hours no fluoresecence could be seen along the epithelial surface of the chicken tracheal rings and only slight fluorescence was noted in duck tracheal rings. From the second day onwards increasing amounts of fluorescent material were seen in both the chick and the duck tissues (Figs.7:30-7:33).

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1 Sigma Chemical Company, St. Louis, MO, U.S.A

Strain *M.gallisepticum* S6LP demonstrated bright fluorescent masses on the luminal epithelial surface of both chick and duck TOCs by 24 hours and by three and five days the fluorescence was more extensive and could be seen as small masses and abundant large masses on the epithelial surface (Figs. 7:34 & 7:35). The fluorescence also extended into the subepithelial layer in some areas. In addition, destruction and detachment of the epithelial cell layer was noted on days three and five. Damaged epithelial cells were also seen at days three and five in strains 4229 and B2/85 but without obvious detachment.

All 3 strains also showed fluorescence around the outside of the tracheal rings on the peritracheal adventia (Figure7:36). It was not seen in the uninfected controls at day 0 but was seen in all infected chick and duck TOCs thereafter except for chick TOCs infected with strains 4229 and B2/85 after 24 hours. Once it had appeared the intensity did not seem to increase with time.

7.2.4 Discussion

These studies have demonstrated that chick and duck embryo TOCs infected with strains 4229 and B2/85 show specific fluorescence due to the accumulation of the organisms along the epithelial cell surface of the tracheal lumen. The picture was similar to that seen with the virulent S6LP strain of *M.gallisepticum* although staining was more intense and extensive with this mycoplasma than with 4229 and B2/85. In addition, strain S6LP demonstrated destruction and detachment of the epithelial cell layer.

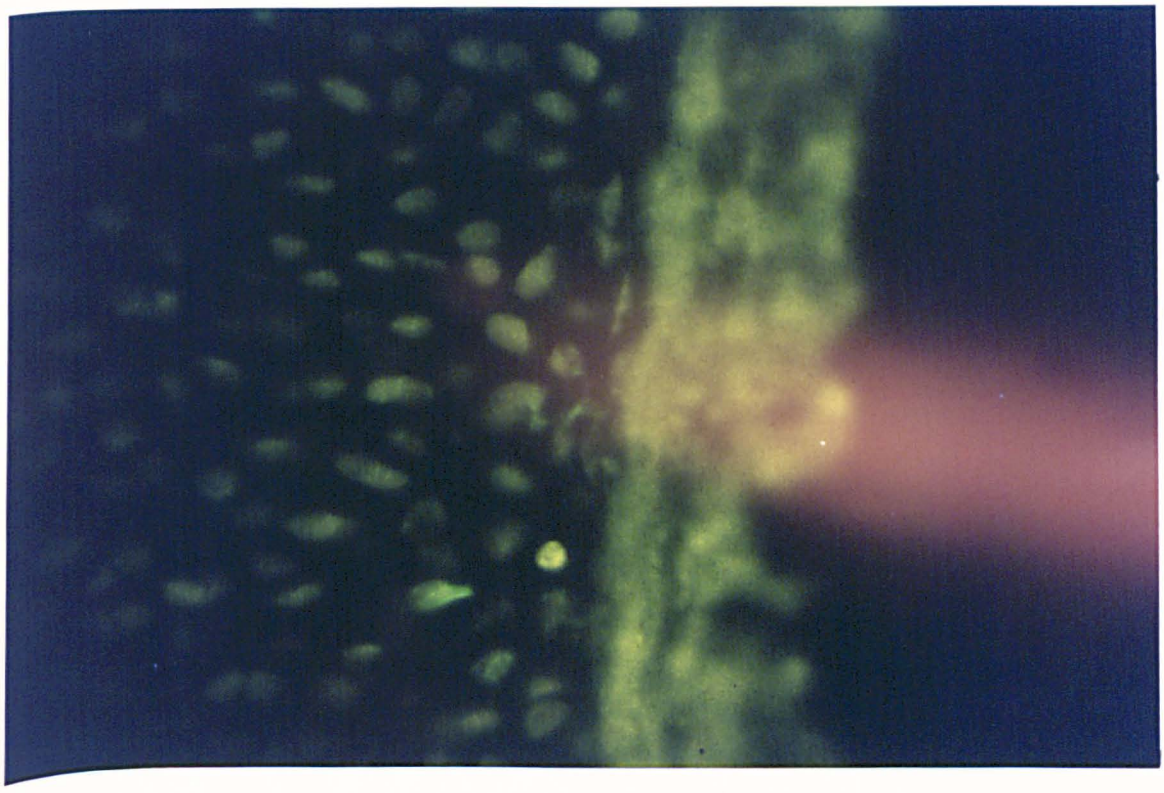


Fig.7:25 Chick embryo TOC control
Infected with strain B2/85 and reacted with normal rabbit serum and conjugate. No specific fluorescence can be seen. x1,600

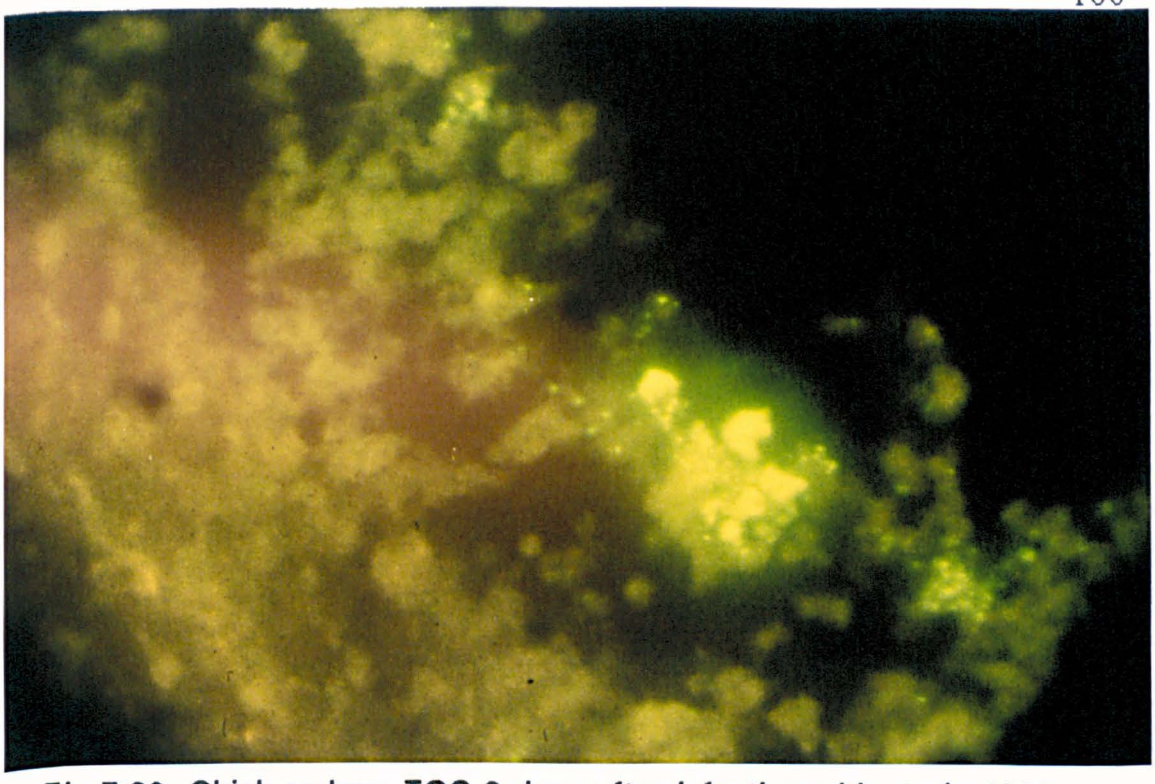


Fig.7:26 Chick embryo TOC 2 days after infection with strain 4229
Patches of bright fluorescence can be seen at the epithelial cell surface. x1,600

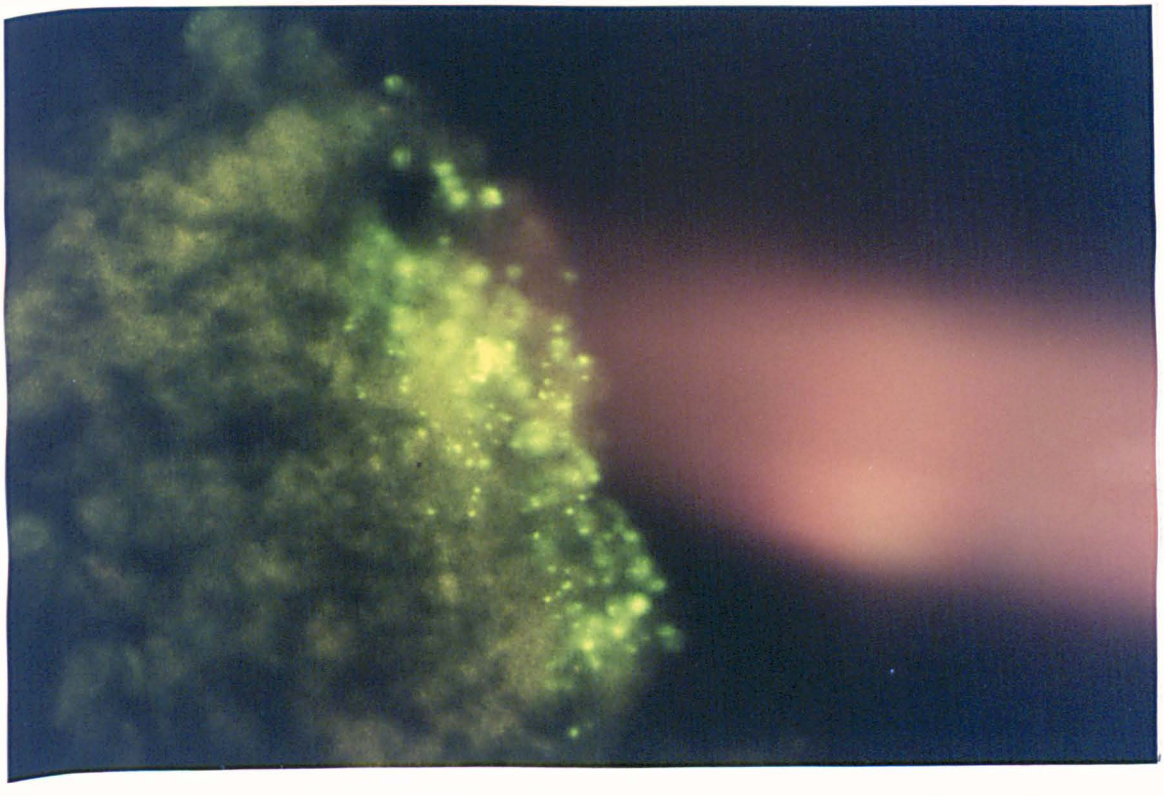


Fig.7:27 Duck embryo TOC 2 days after infection with strain 4229
Patches and granules of bright specific fluorescence at the epithelial surface. x1,600.

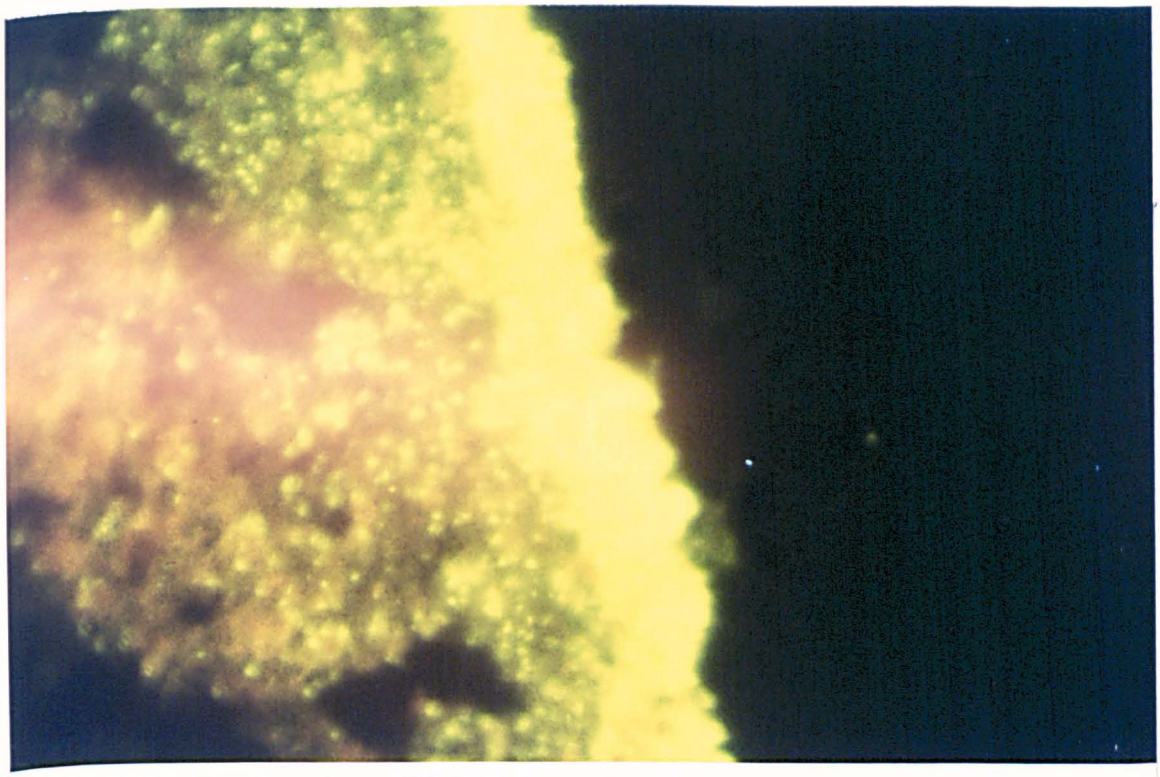


Fig.7:28 Duck embryo TOC 3 days after infection with strain 4229
Specific fluorescence extends into the subepithelial layer. x1,600.

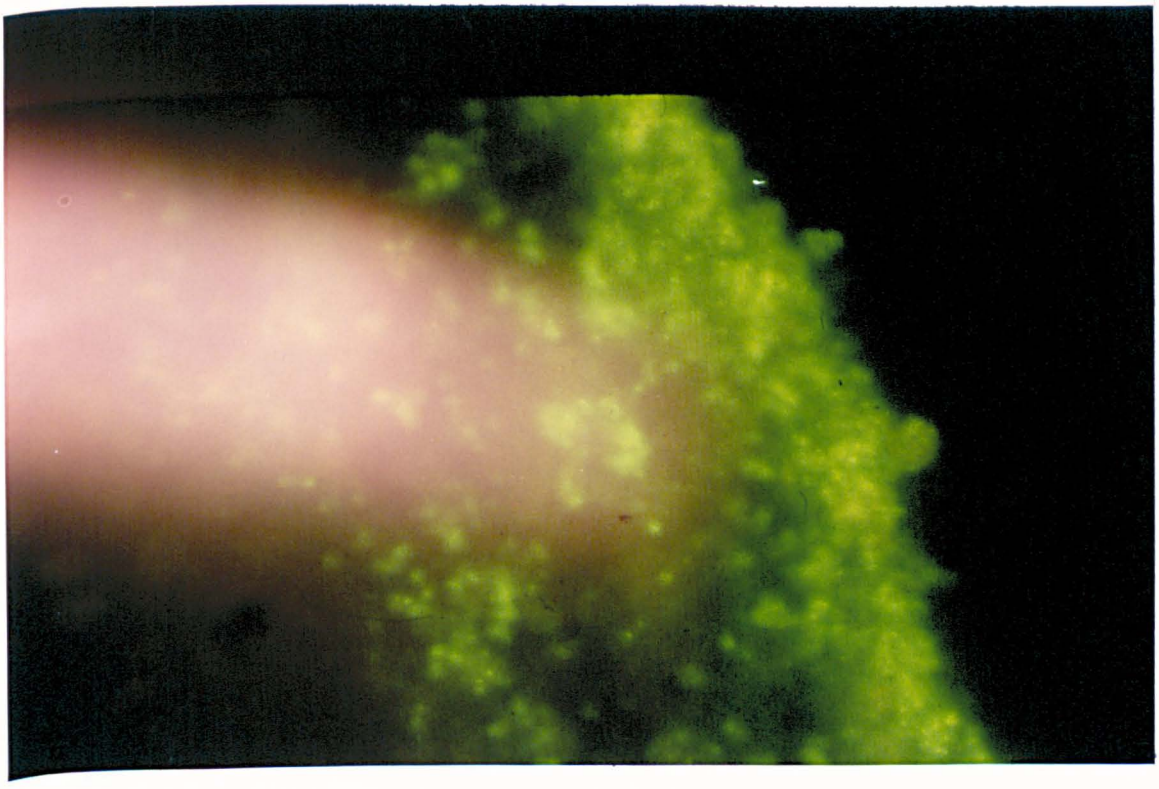


Fig.7:29 Chick embryo TOC 5 days after infection with strain 4229
Specific fluorescence extends into the subepithelial layer. x1,600.

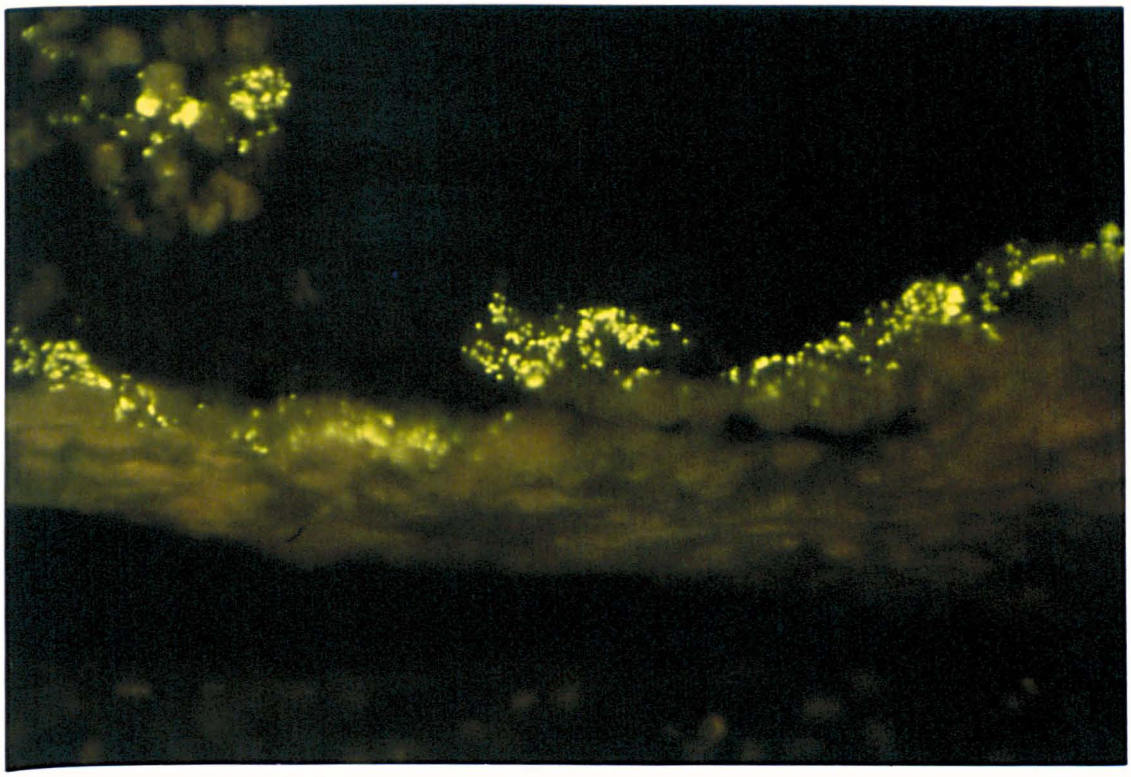


Fig.7:30 Chick embryo TOC 2 days after infection with strain B2/85
Granules of specific fluorescence can be seen at the epithelial surface. x1,600.

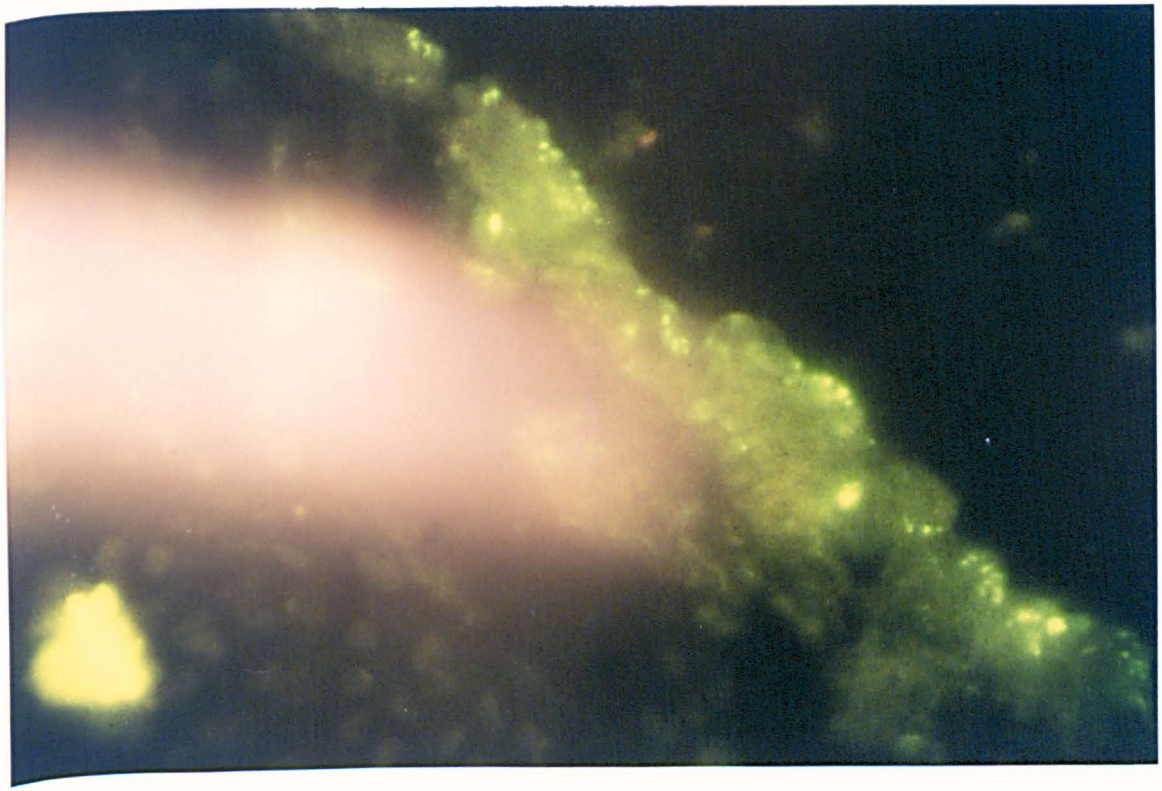


Fig.7:31 Duck embryo TOC 2 days after infection with strain B2/85
Granules of specific fluorescence can be seen at the epithelial surface. x1,600.

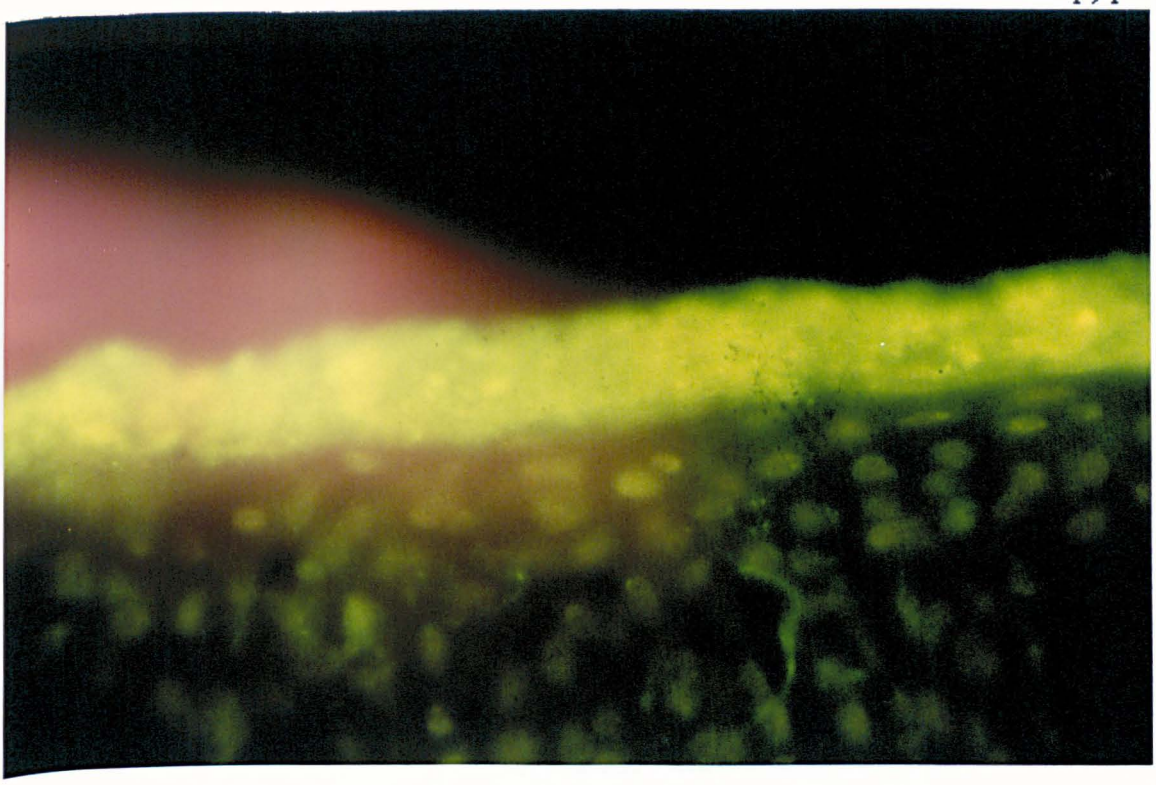


Fig.7:32 Chick embryo TOC 5 days after infection with strain B2/85
Heavy specific fluorescence can be seen at the luminal border. x1,600

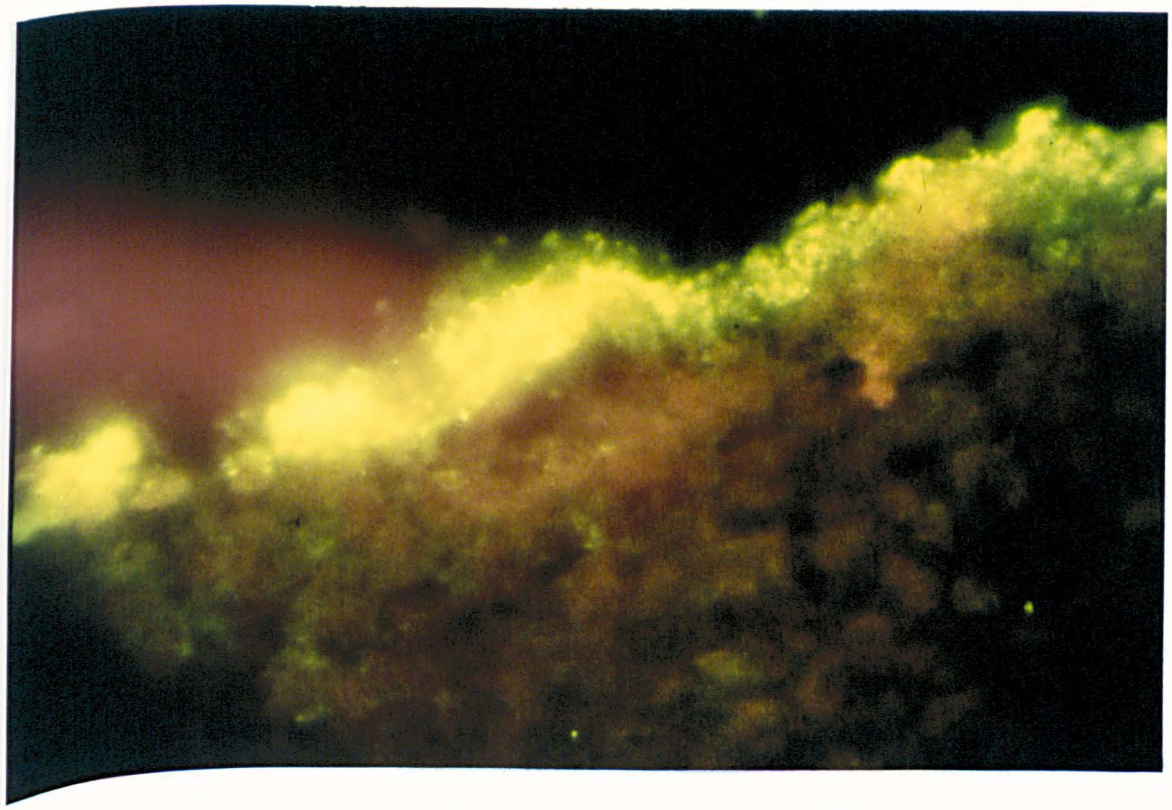


Fig.7:33 Duck embryo TOC 5 days after infection with strain B2/85

Heavy granular specific fluorescence can be seen at the luminal border. x1,600

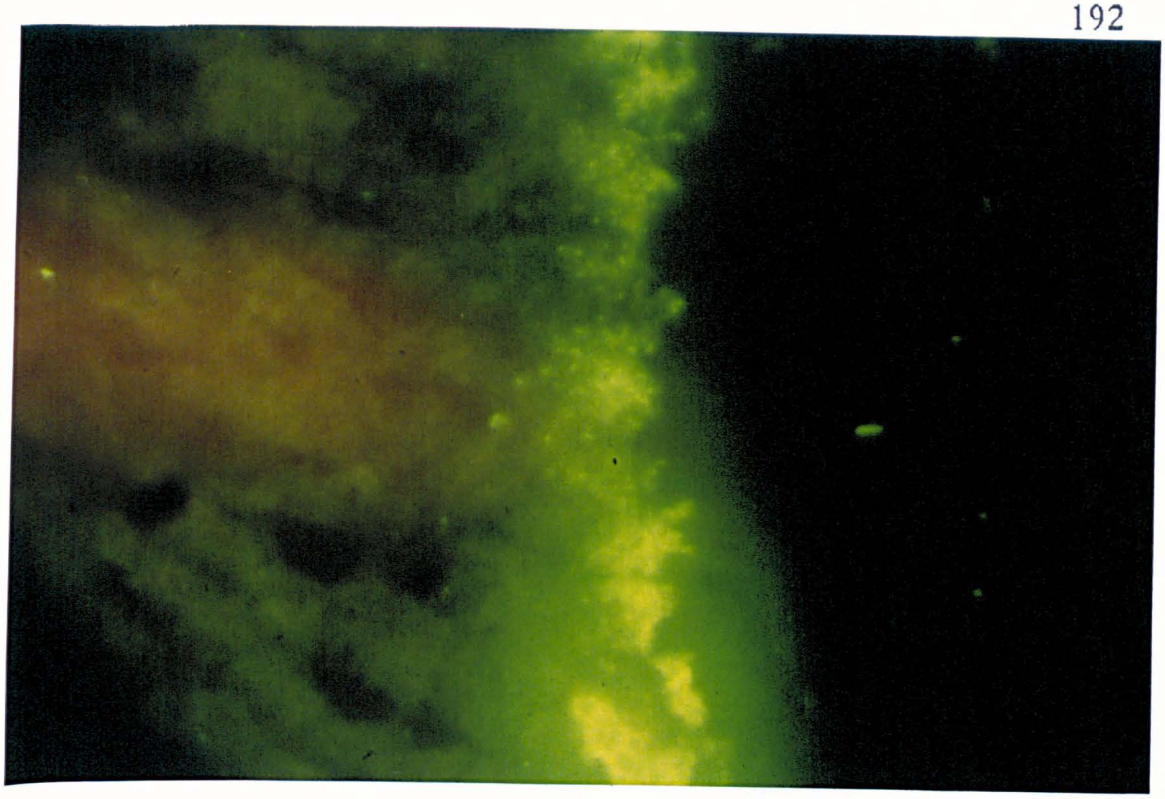


Fig.7:34 Duck embryo TOC 3 days after infection with strain S6LP
Patches and granules of specific fluorescence can be at the luminal border. x1,600.

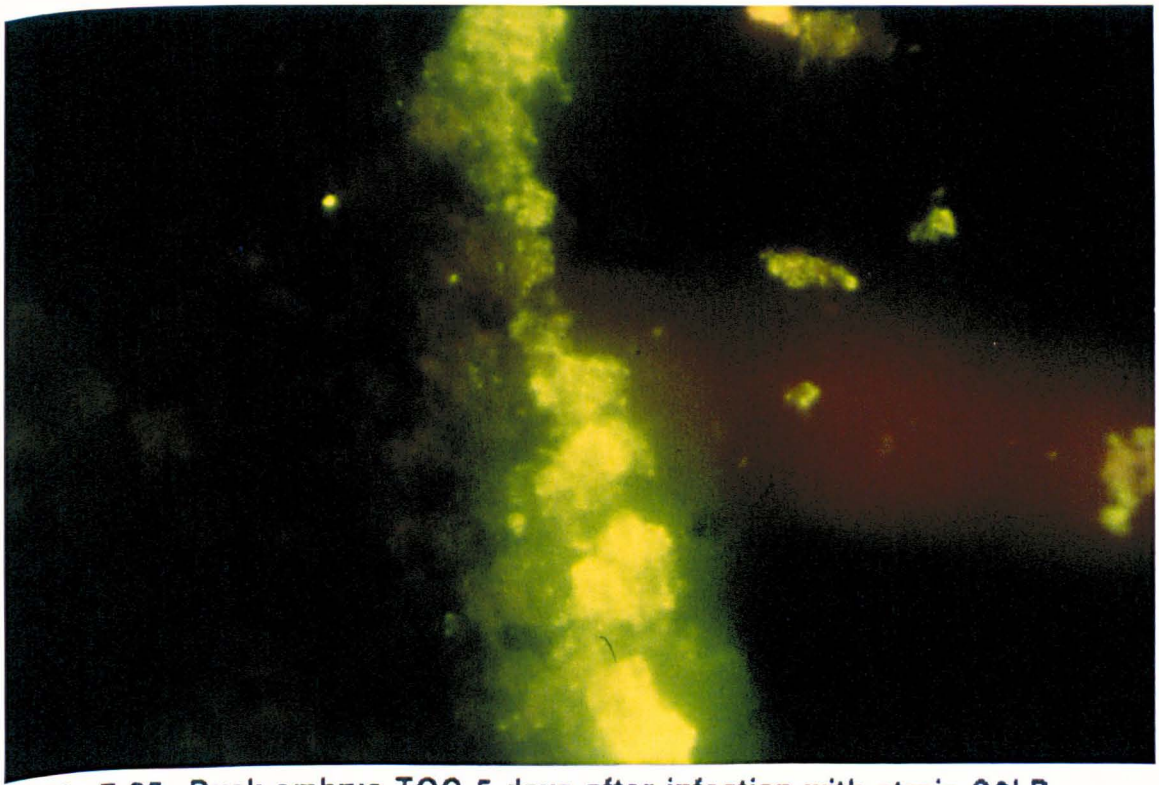


Fig.7:35 Duck embryo TOC 5 days after infection with strain S6LP
Heavy patches of specific fluorescence can be seen at the luminal border. x1,600

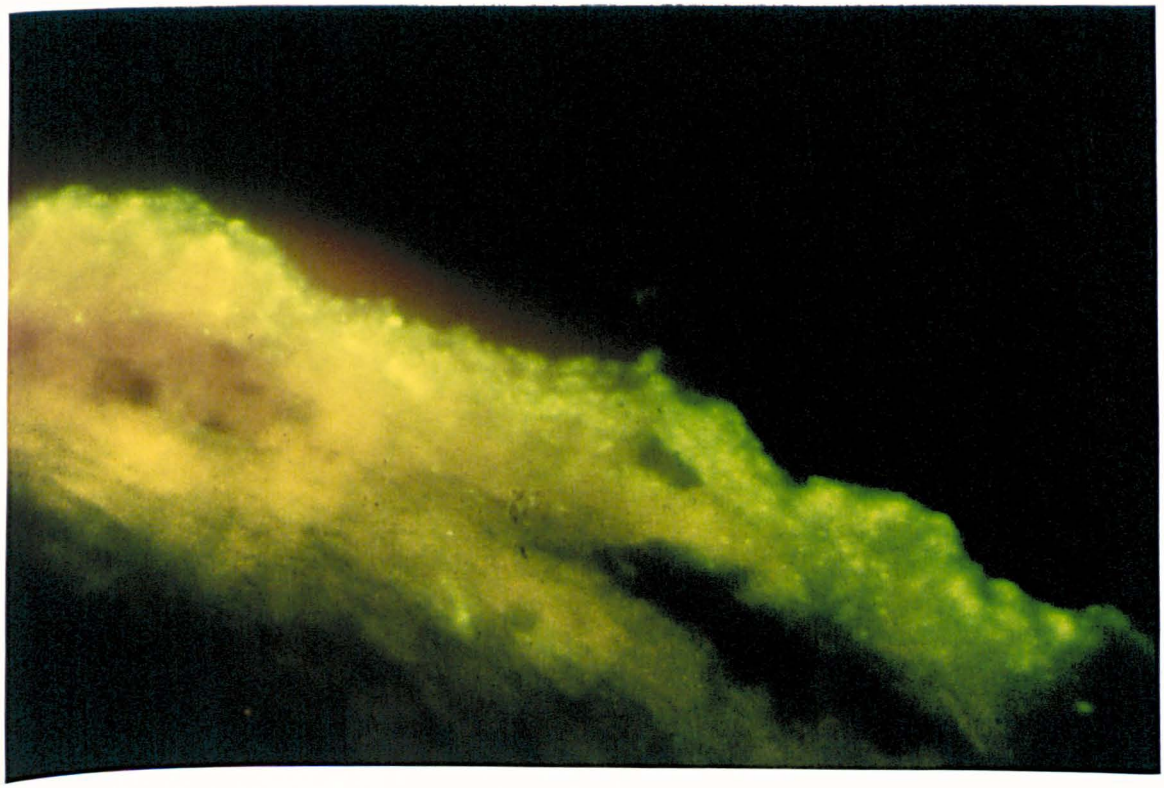


Fig.7:36 Chick embryo TOC 2 days after infection with strain 4229

There is an accumulation of fluorescent material on the periphery of the peritracheal adventitia. x1,600

These findings indicate that the inhibition of ciliary activity seen in infected chick and duck embryo TOCs and the damage to the epithelium may be a consequence of increasing accumulation of the test organisms at the epithelial cells, but the mechanisms of pathogenicity are not understood. It is thought that the direct damage of infected respiratory tissue in mycoplasma infections can explain only the ciliostasis and epithelial cell losses and may be only a minor component of the total disease picture (Cassell *et al.*, 1985).

The interaction between the test organisms and the epithelial cells could not be defined adequately using immunofluorescence, hence the use of electron microscopy to provide further information. Collier and Clyde, (1971) demonstrated by immunofluorescence the localization of *M.pneumoniae* in relation to the damaged epithelial cells of human foetal TOC. They found that the organisms were concentrated on the luminal surface of the ciliated epithelium and cells lining the submucosal glands. However, the exact relationship between the individual organisms and ciliated epithelial cells could not be elucidated by immuno-fluorescence, and electron microscopy provided further information at the cellular level (Collier and Clyde, 1971; Collier *et al.*, 1971). Both *M.synoviae* WVU 1853 and *M.gallisepticum* S6 were observed by immunofluorescence on the luminal surface of chick embryo tracheal organ cultures but, when examined by electron microscopy, *M.synoviae* was found only near the cilia while *M.gallisepticum* was attached to the epithelial cells (Hirano *et al.*, 1978).

Our immunofluorescence studies indicated that strains 4229, B2/85 and S6LP not only showed specific fluorescence on the luminal epithelial cell surface but also on the peritracheal ring adventitia at almost all the time intervals after inoculation. Gabridge *et al.* (1979) stated that mycoplasmas can 'bind non-specifically' to the outer surface of the trachea but did not present any experimental data. The finding of fluorescence at this location does not appear to have been reported by other workers

with the exception of a study on infection of chick embryo TOCs with *M.gallisepticum* and *M.synoviae* by Oriel (1976). The fluorescence was considered to be specific rather than non-specific since none was seen in the negative control preparations, including uninfected tracheal rings reacted with antiserum and conjugate. Therefore, peritracheal fluorescence was thought to indicate the accumulation of inoculated organisms at this site although, compared with the observations on the luminal surface, the organisms did not appear to multiply. In her study Oriel (1976) suggested the possibility that the organisms became physically trapped in the peritracheal adventitia. As mentioned earlier in this Chapter (7.1.4), such binding could be a drawback for studies on pathogen uptake by introducing errors (Gabridge, 1979; 1984).

It would have been useful to confirm the occurrence of the organisms in the peritracheal location by electron microscopy but in our studies the ultrathin sections were confined to the luminal area and time did not allow us to prepare further sections.

The immunofluoresence observations of the test strains in both chicken and duck TOCs correlated with the growth of these organisms (from 1 up to 5 days) since increasing intensity of fluorescence was seen in the tracheal lumen along with an increase in the titres of viable organisms, which attained maximum titres of 10^6-10^7 CFU/ml by days 6-10 in chick embryo TOCs and 10^5-10^6 CFU/ml by days 4-6 in duck TOCs.

In conclusion, immunofluorescent staining of strains 4229 and B2/85 and *M.gallisepticum* S6LP in chicken and duck embryo TOCs showed the organisms on the epithelial surfaces in close association with epithelial cells indicating the possibility of cytadherence of strains 4229 and B2/85 to the tracheal epithelium as is seen with *M.gallisepticum* (Abu-Zahr and Butler, 1976; Uppal and Chu, 1977; Tajima

et al., 1979). The electron microscopy studies were carried out to provide more precise detail of the mycoplasma-host cell relationship.

7.3 Transmission electron microscopy studies

7.3.1 Introduction

Most pathogenic mycoplasma species of human and animals appear to be extracellular organisms having the epithelial lining of mucosal surfaces as their target cells. The large area of mucosal surfaces in the host frequently causes difficulty in locating and examining the interaction of the individual organisms and their target cells, and the use of organ culture techniques has helped workers to locate the organisms by enabling the maintainence of small pieces of differentiated, organised epithelial mucosa *in vitro* (Collier and Carson, 1983).

Electron microscopy has been used to examine the relationship between the individual organisms and the host tissue in organ culture and has included both transmission and scanning microscopy. In the former the ultrathin sections permit study of the internal ultrastructure of both the organisms and host cell, but allow only small areas of the sample to be thoroughly examined, whereas use of the scanning electron microscope allows a large surface area to be examined but is restricted to the external surfaces (Collier, 1979).

Association between various mycoplasmas and TOCs has been investigated by a number of workers using electron microscopy. For example chicken embryo TOCs have been examined after inoculation with *M.gallisepticum* S6 (Hirano *et al.*,1978), or with a low passage culture of the virulent R strain of *M.gallisepticum* (Dykstra *et al.*, 1985). Hamster TOCs (Collier *et al.*, 1971; Muse *et al.*, 1976) and human foetal TOCs (Collier and Clyde, 1971) have been examined after inoculation with a virulent strain of *M.pneumoniae* , and mouse TOCs after inoculation with *M.pulmonis* (Araake, 1982). Tracheal organ cultures of rats and piglets have also been studied after infection with *M.mobile* (Stadtlander and Kirchoff, 1988; 1990).

Transmission and scanning electron microscopy were used to study the parasitic mode of *M.gallisepticum* and *M.gallinarum* in chick embryo tracheal explants by Abu-Zahr and Butler (1976) who showed that *M.gallisepticum* first colonised the epithelium then later the *lamina propria*, forming a close association with the cells, whereas *M.gallinarum* appeared first in the *lamina propria* and did not develop an intimate association with cells.

One of the important aspects of our electron microscopy study of strains 4229 and B2/85 in chicken and duck TOCs was to obtain further information on the specialised organelle that was seen in the cell pellets and which resembled the terminal tip-like structure of the closely-related *M.gallisepticum*. It was of interest to examine the organisms in infected cells to see if these structures appeared to be associated with adherence, as the contact site between the organisms and the host cells (Maniloff *et al.*, 1965; Zucker-Franklin *et al.*, 1966).

The possible role of the bleb-like organelles in *M.gallisepticum* adherence was investigated by electron microscopy of infected fowl tracheal epithelium and it was found that the mycoplasmas often appeared to be attached to the membrane of the epithelial cells by their blebs (Uppal and Chu, 1977). The authors suggested that these blebs might represent suckers which allow attachment of the mycoplasma to the host cells, thus enhancing pathogenicity. Slight to moderate changes were observed in the epithelial cell structure of the infected birds including loss of cilia and slight enlargement of mitochondria and endoplasmic reticulum.

The purpose of our electron microscope study was therefore to elucidate the interaction of strains 4229 and B2/85 with the tracheal epithelium of the chicken and duck *in vitro*, to compare with that of *M.gallisepticum* S6 and to observe the possible relationship of the tip-like structure to cytadherence.

7.3.2 Materials and Methods

Mycoplasma strains

Strains 4229, B2/85 and *M.gallisepticum* S6LP were used as before (Table 7:1).

Tracheal organ cultures

Tracheal organ cultures were prepared from chick and duck embryos as described previously (7.1.2). OCM without serum was used, and the preparation and administration of the three mycoplasma inocula were as described for immunofluorescence (7.2.2).

Electron microscopy

The method used was that of Dykstra *et al.* (1985).

Materials:

McDowell and Trump's fixative (McDowell and Trump, 1976)

Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	1.16 g
Sodium hydroxide	0.27 g
Glutaraldehyde (50%)	2.0 ml
Formaldehyde (40%)	10.0 ml
Distilled water	88.0 ml

The solution was stored at 4°C for up to 3 months.

Procedure:

Duplicate tracheal rings were taken from chick and duck embryo TOCs at 0 days (uninoculated controls) and 1, 2, 3 and 5 days after inoculation. They were immersed in McDowell and Trump's fixative, and stored for 24 hours at 4°C. The fixative was then removed and the tissue washed in 0.1 M phosphate buffer (pH 7.2). The washing buffer was discarded and secondary fixation (post-fixation) in 1% osmium tetroxide (4.2.2) in the same buffer was performed at room temperature for 30 minutes to one hour. The post-fixative was discarded and the tissues were washed in deionized water. All the procedures for dehydration in acetone, embedding in Spurr's resin, preparation of ultrathin sections and staining were as described before (4.2.2).

7.3.3 Results

Ultrathin sections of uninfected tracheal rings of both chicken and duck embryo organ cultures had epithelial cells with cilia and microvilli (Figs. 7:37 and 7:38). Some non-ciliated cells were also observed.

In sections prepared from chick and duck TOCs infected with strains 4229 and B2/85 there was loss of cilia and the microvilli were reduced in number. This was seen within two days with strain 4229 whereas with B2/85 most cilia and microvilli still remained intact on day two. Three and five days after infection there was further loss of cilia and the microvilli were fewer in number and shorter in length.

In these sections strains 4229 and B2/85 had the same basic ultrastructural morphology as they had in the pellets prepared from broth cultures (4.3). Many cells were of characteristic pear-shape when sectioned through their longitudinal axis and others showed a rounded shape. Other morphological features included the

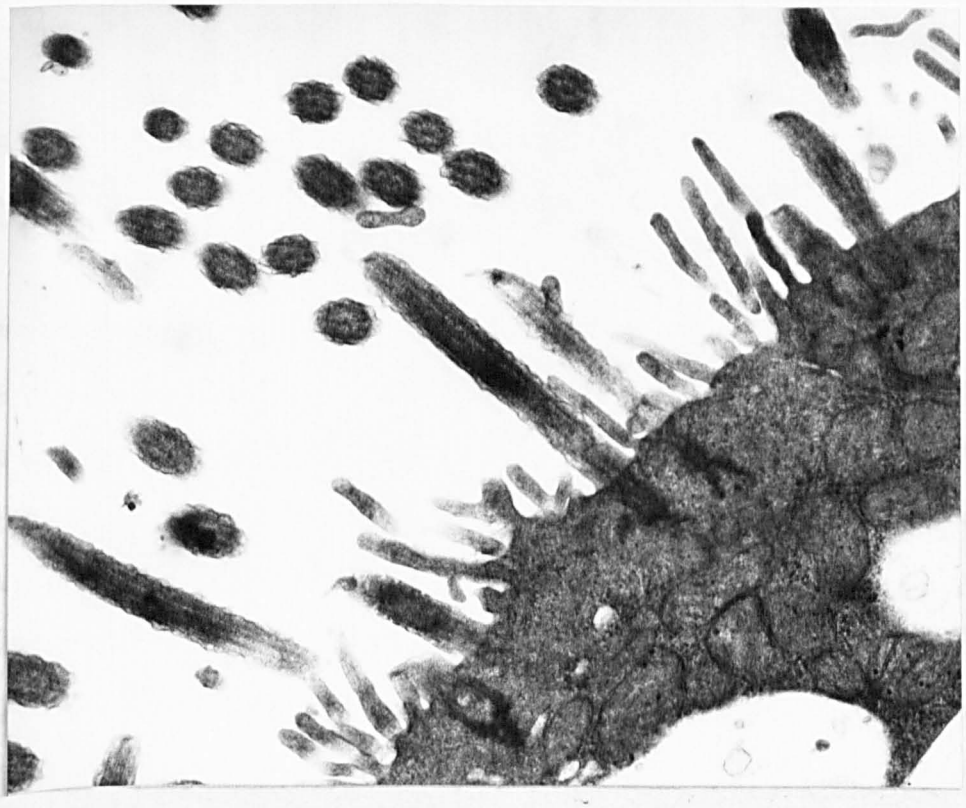


Fig.7:37 Electron micrograph of uninfected chick embryo TOC
Intact cilia and microvilli can be seen. Many cilia have been sectioned horizontally. x24,000.

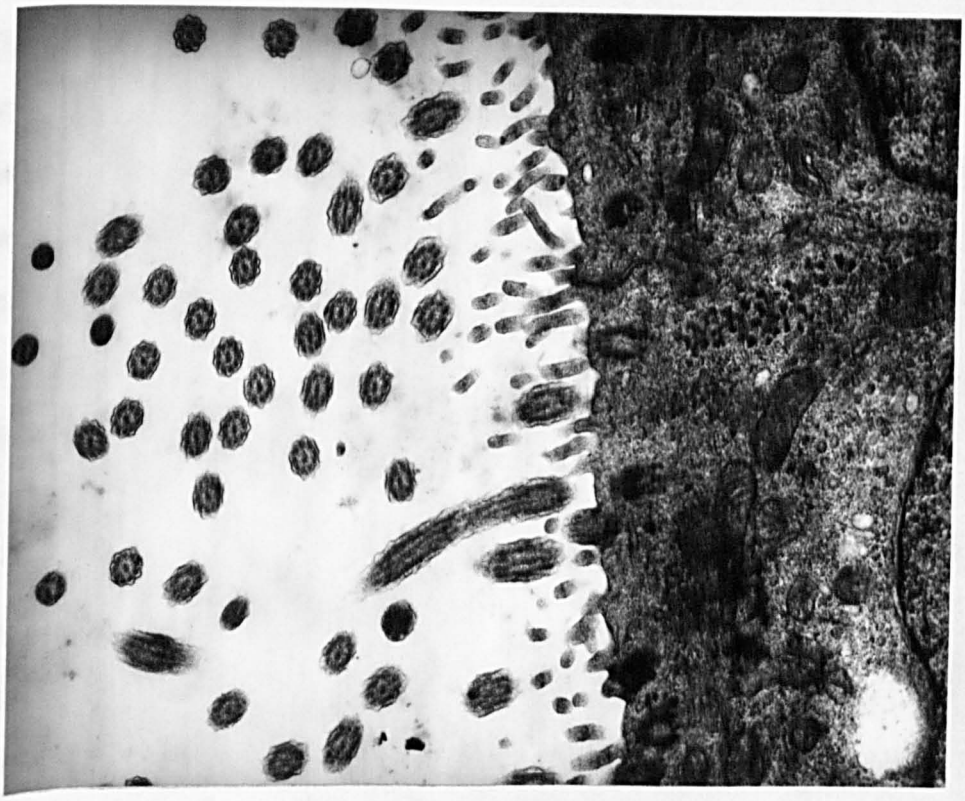


Fig.7:38 Electron micrograph of uninfected duck embryo TOC
Numerous intact cilia and microvilli can be seen. x17,000.

triple-layered membrane and absence of a cell wall. There were no obvious structural differences noted between the two strains.

With both strains many organisms were seen in close proximity to the epithelial surface between the cilia or microvilli, with others lying free in the lumen. Many of the organisms appeared to be attached to the epithelial cell surface, and some to the microvilli or cilia, and the attachment appeared to be through their terminal tip-like structure (Figs. 7:39-7:42). Attached organisms were seen on both ciliated and non-ciliated cell surfaces. The tip-like organelles appeared to consist of a dense central core surrounded by moderately dense material and this was covered externally by the triple-layered membrane. No mycoplasmas were seen within the epithelial cells or between them.

A similar deciliated epithelial surface was evident in both the chick and duck embryo TOCs infected with *M.gallisepticum* S6LP but the damage was more severe and deciliation was followed by detachment of fragments of epithelium into the lumen. These fragments, which had mycoplasmas attached to them, were seen by day 3 and were still evident on day 5. As with the other strains most of the organisms in close proximity to the epithelial surface of both ciliated and non-ciliated cells were attached via their terminal tip (Figs. 7:43-7:46). The morphology of these organisms appeared very similar to that of strains 4229 and B2/85.

All 3 strains appeared as a rounded form when they were more distant from the surface epithelium of both chicken and duck tracheal rings whereas, when they were close to the epithelium, they exhibited pear-shaped morphology with attachment organelles which were directed towards the epithelial surface. The mycoplasmas could be observed as either single organisms or clusters, and in those that were attached the tip-like organelles were pushed down onto the epithelial surface. With

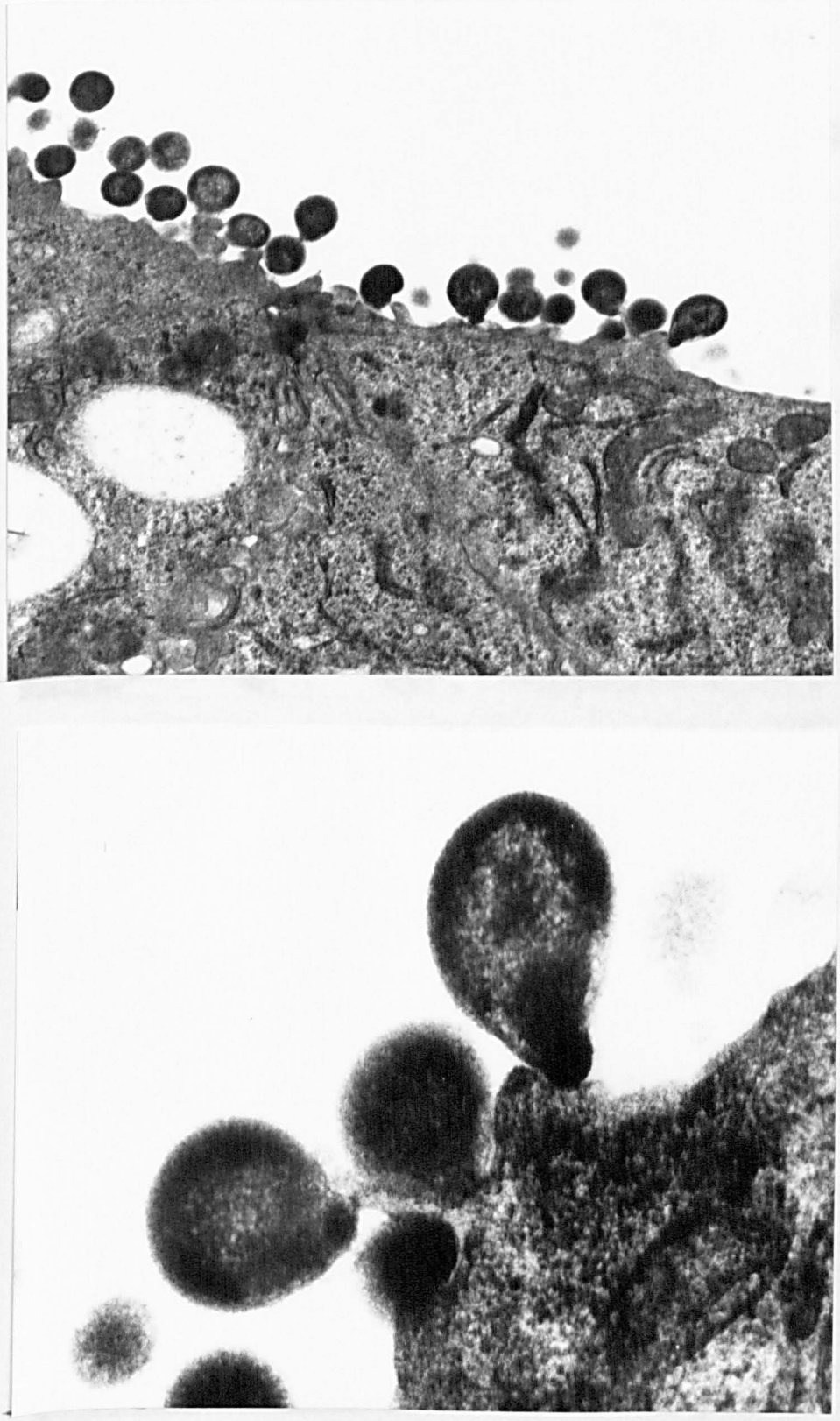
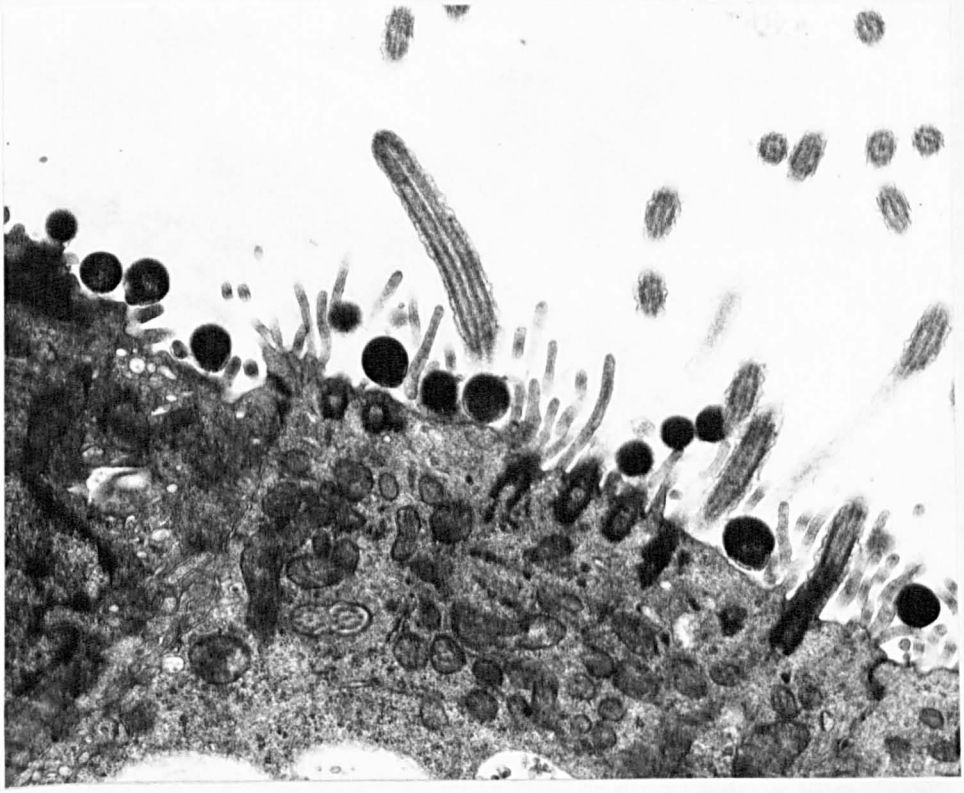


Fig.7:39 Electron micrographs of chick embryo TOC 2 days after infection with strain 4229

A. Mycoplasma cells appear as rounded or elongated electron-dense structures at the cell surface. x16,000

B. The terminal tip appears to be pushed down onto the epithelial cell surface. x24,000.

A



B

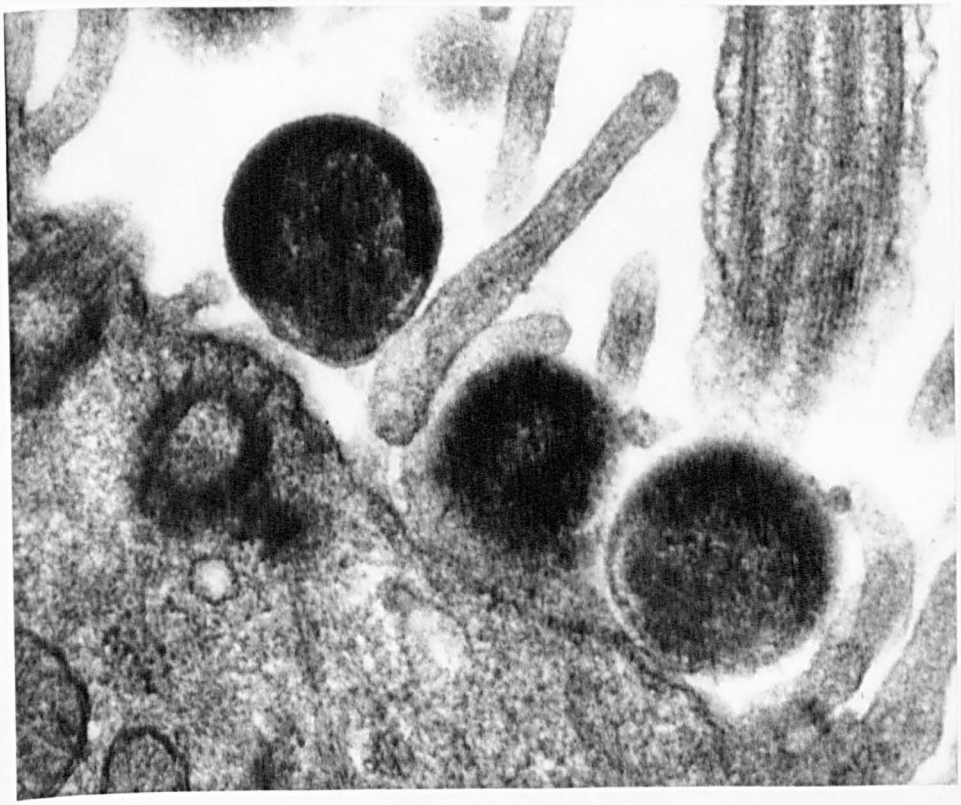
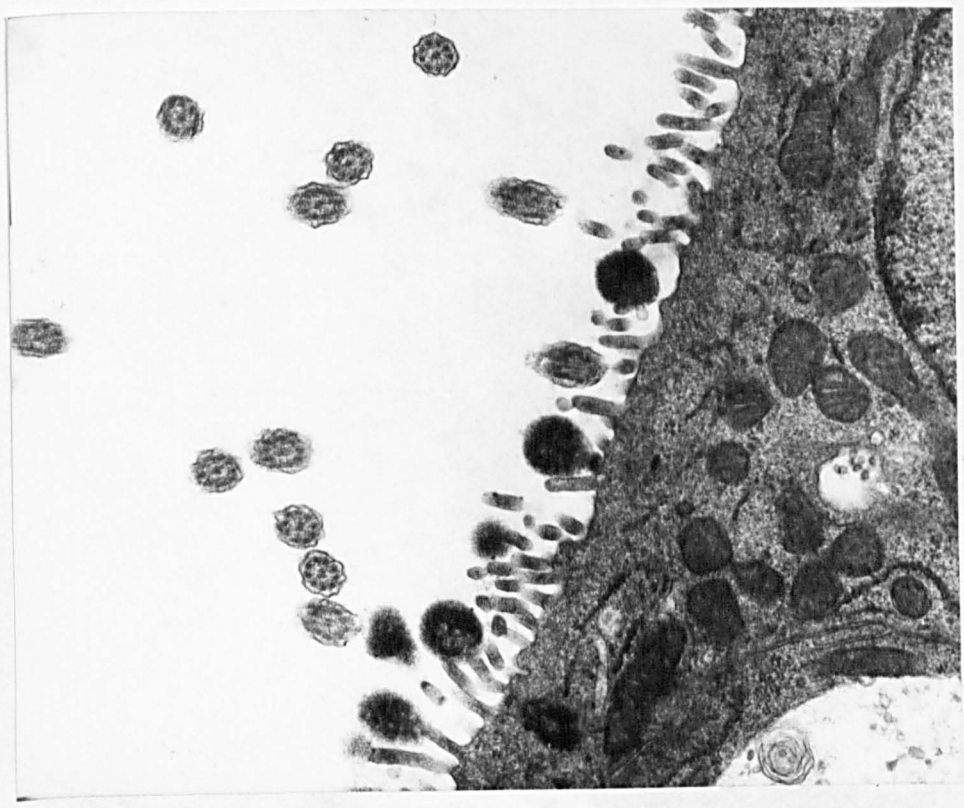


Fig.7:40 **Electron micrographs of duck embryo TOC 2 days after infection with strain 4229**

A. Mycoplasma cells appear as rounded or elongated electron-dense structures between the microvilli at the cell surface. x15,000

B. The terminal tip appears to be in close proximity to the epithelial cell. surface. x70,000.

A



B

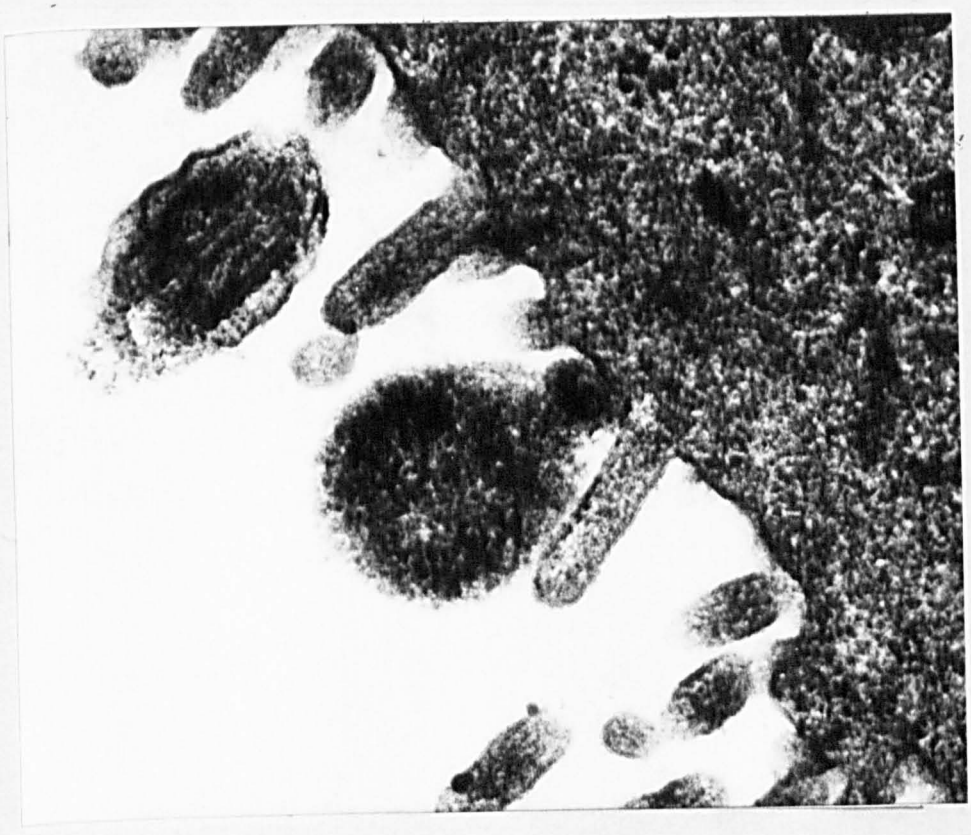
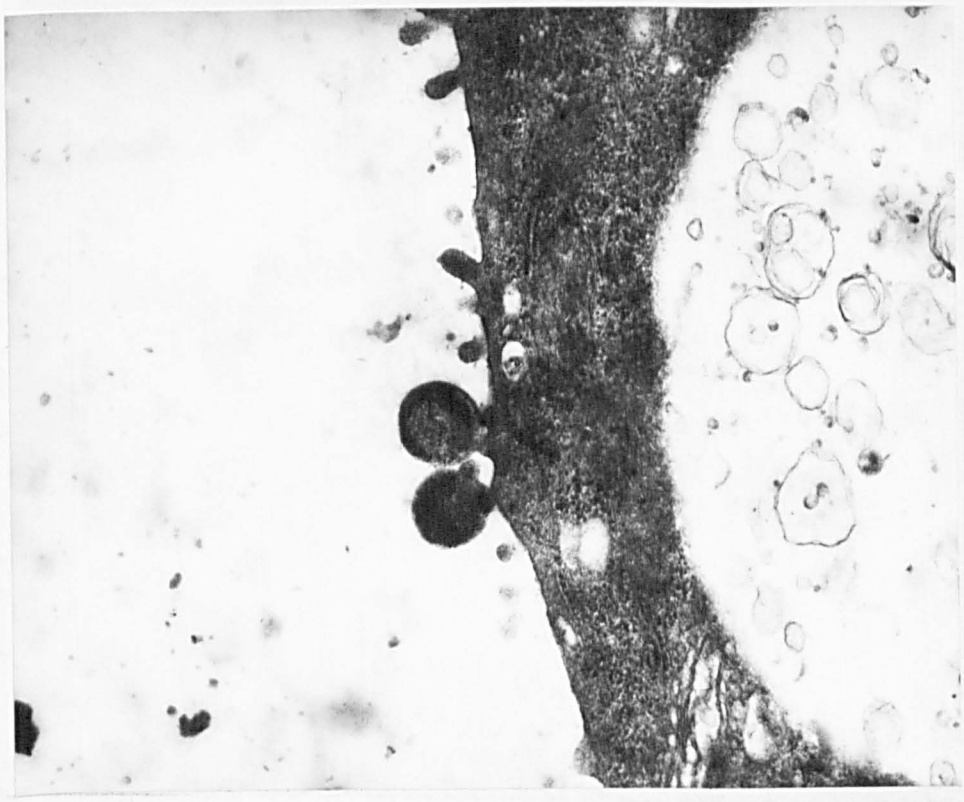


Fig.7:41 **Electron micrographs of chick embryo TOC 2 days after infection with strain B2/85**

A. Mycoplasma cells appear as rounded or elongated electron-dense structures between the microvilli at the cell surface. x17,000

B. A well defined terminal tip stucture can be seen adjacent to the epithelial cell membrane. x85,000.

A



B

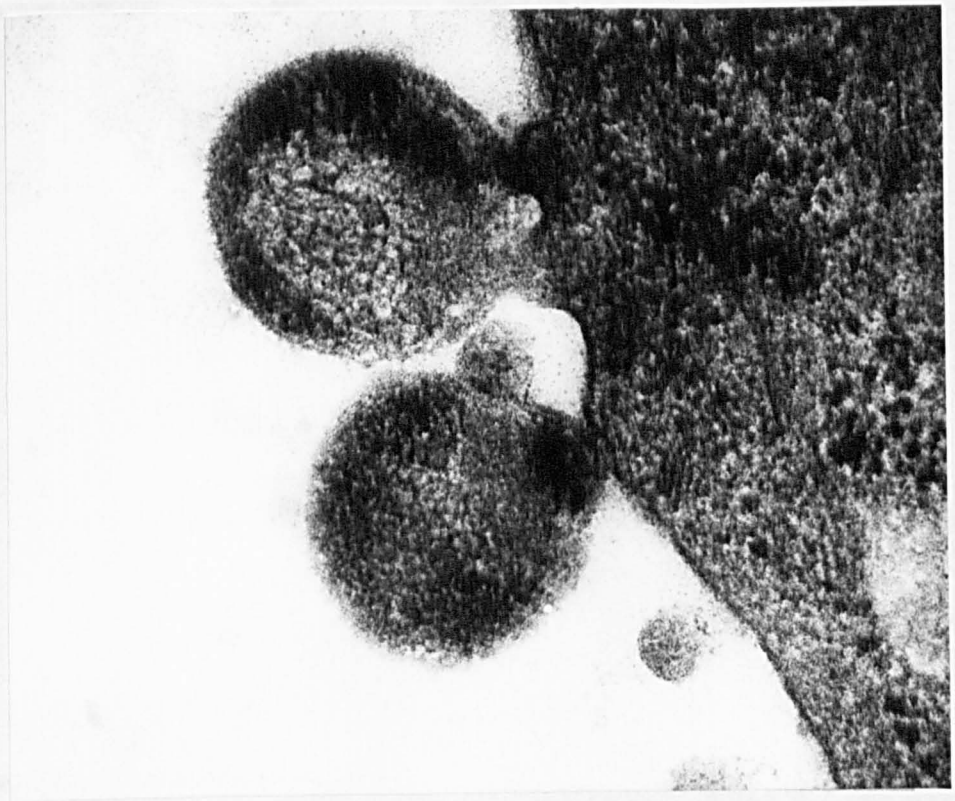
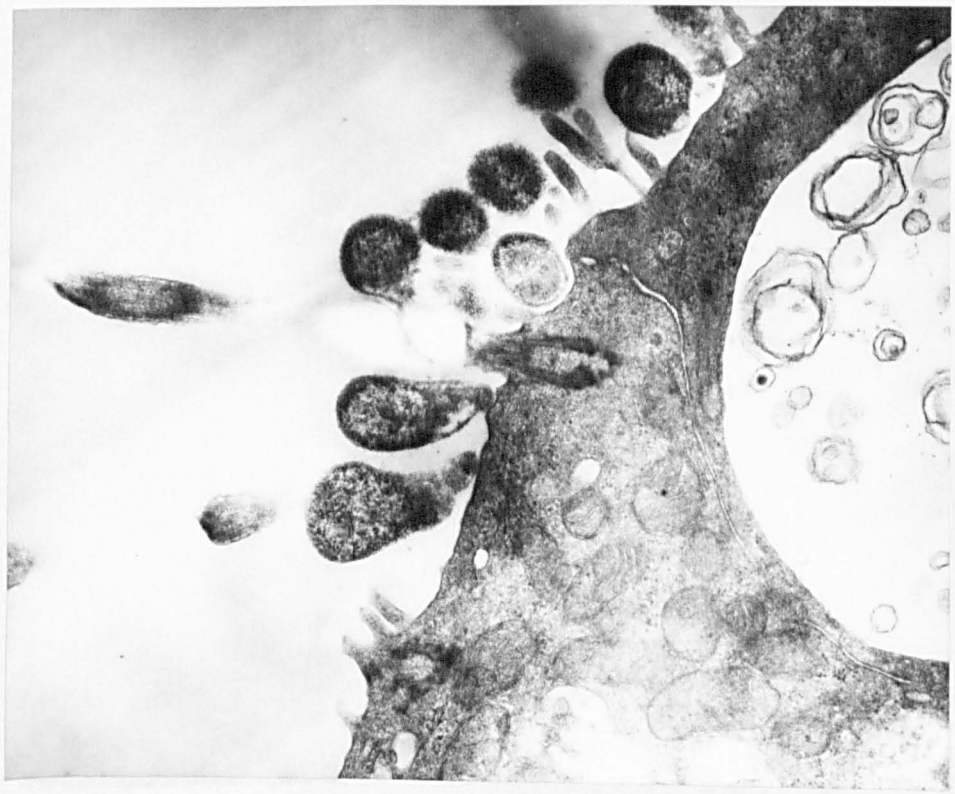


Fig.7:42 **Electron micrographs of duck embryo TOC 2 days after infection with strain B2/85**

A. Two mycoplasma cells appear as electron-dense structures at the cell surface. x28,000

B. The terminal tips of both cells appear to be pushed down onto the epithelial cell membrane. x114,000.

A



B

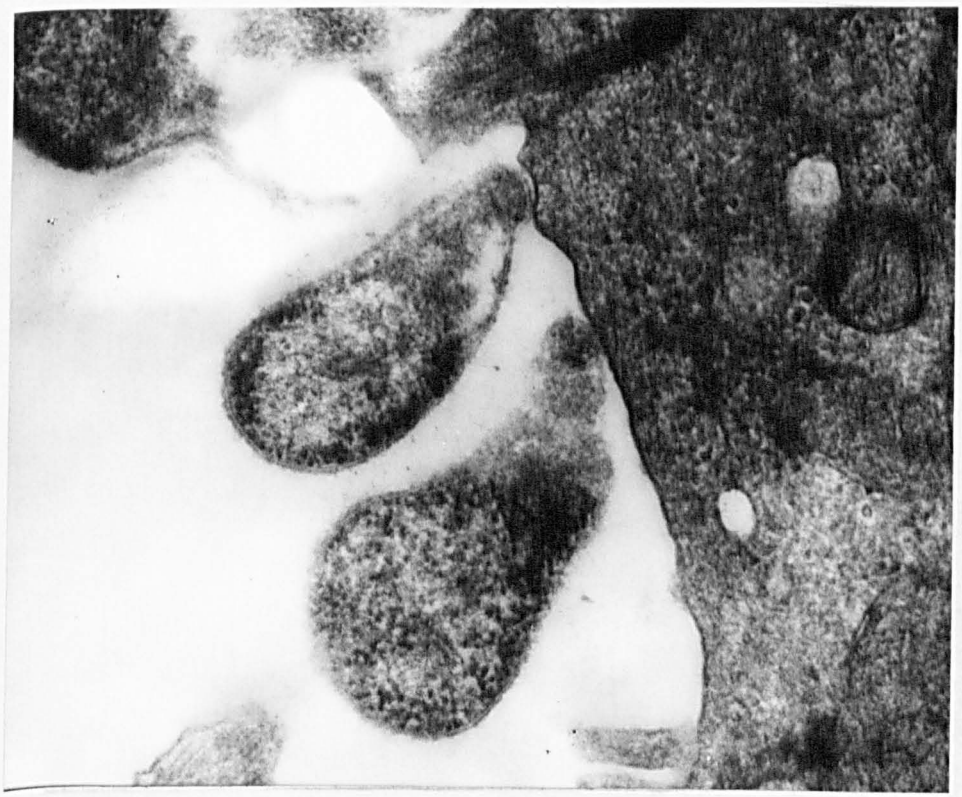
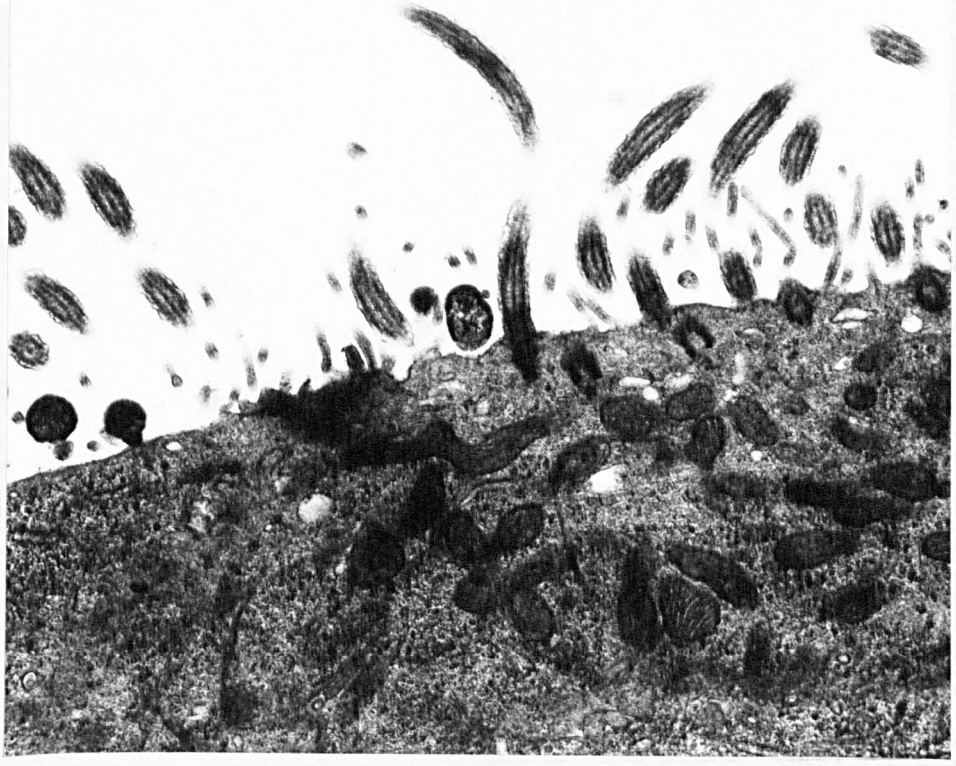


Fig.7:43 **Electron micrographs of chick embryo TOC 2 days after infection with *M.gallisepticum* S6LP**

A. Mycoplasma cells appear as rounded or elongated electron-dense structures at or near the cell surface. x28,000

B. With one of the organisms the terminal tip appears to be pushed down onto the epithelial cell. x70,000.

A



B

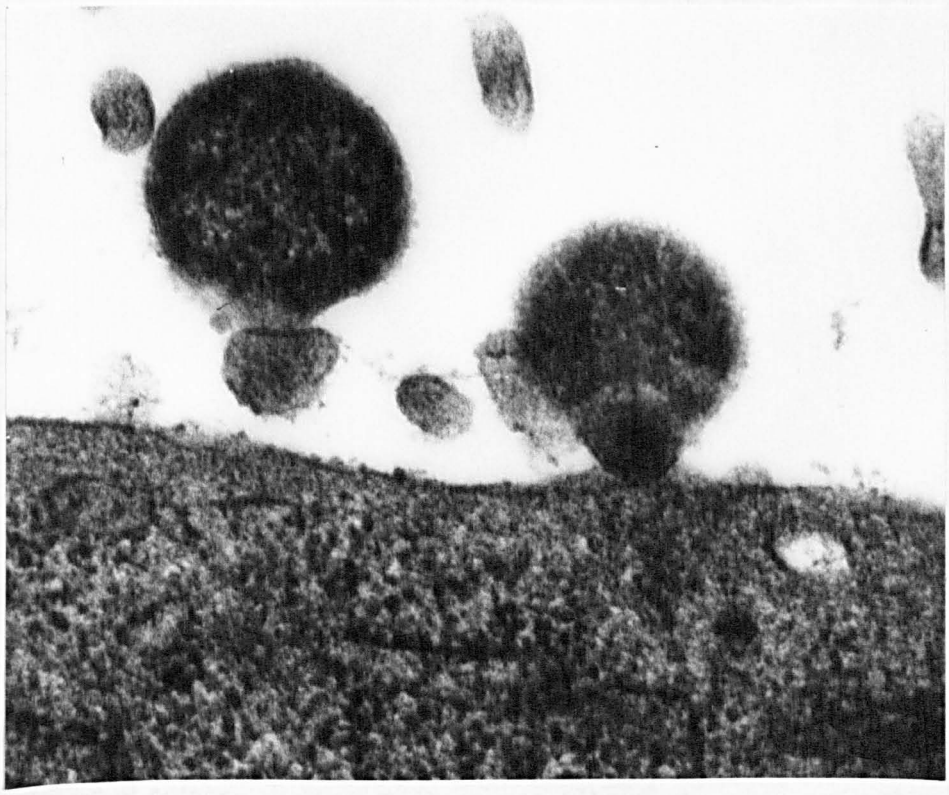


Fig.7:44 **Electron micrographs of duck embryo TOC 2 days after infection with *M.gallisepticum* S6LP**

A. Two mycoplasma cells appear as elongated electron-dense structures and a third has a rounded appearance. x16,000

B. The terminal tip of one organism appears to be pushed down onto the epithelial cell membrane. x85,000.

other organisms attachment between the mycoplasmas and epithelial surface was impossible to see and there was a space between them.

7.3.4 Discussion

Deciliation was the most prominent feature seen in the chick and duck embryo TOCs infected with strains 4229, B2/85 and S6LP and examined by electron microscopy in a small number of serial sections at various times intervals after inoculation. The time intervals of 1, 2, 3 and 5 days were chosen in an attempt to locate the organisms during any attachment process that might occur and hence to demonstrate the possible role of the terminal tip-like structures.

Although some epithelial cells in the uninfected tracheal control rings did not have cilia, most cells possessed intact cilia and microvilli, and there was a clearly visible difference between uninfected and infected tracheal rings at the cellular level. However the uninfected controls were examined only at day 0 and ideally they should have been tested at each of the times that the infected samples were examined. The finding of non-ciliated areas in uninfected tracheal rings is in agreement with Dykstra *et al.* (1985) who observed slightly larger non-ciliated areas in control tracheal samples examined at day 3 compared to day 0.

The interaction between strains 4229 and B2/85 and the tracheal epithelium of both chick and duck embryo organ cultures resulted in less severe damage than that caused by *M.gallisepticum* S6LP, in which deciliation of the epithelium was followed by exfoliation of patches of the epithelium with attached mycoplasmas into the lumen. Nevertheless the damage caused by strains 4229 and B2/85 indicated their pathogenicity for both chick and duck embryo tissue and correlated with the ciliostasis and the immunofluorescent staining reported above.

Loss of cilia and destruction of the epithelial cells by *M.gallisepticum* agrees with the observations of other workers (Abu-Zahr and Butler, 1976) and the exfoliation of the epithelium, with mycoplasmas attached, has been demonstrated by these authors as well as Tajima *et al.* (1979) and Dykstra *et al.* (1985).

The ultrastructure of strains 4229 and B2/85 in the tissue sections confirmed that both have a specialised tip-like organelle at one end and that it appears to mediate attachment to the epithelial cell surface of both avian hosts in a similar manner to the bleb-like attachment organelle of *M.gallisepticum*. This study also showed for the first time that *M.gallisepticum* attaches to duck tracheal epithelium.

There is, as yet, no other information available on the possible pathogenetic mechanisms of strains 4229 and B2/85. However we discuss here some factors that might be involved, in the light of information obtained from studies with other mycoplasmas. Due to the close relationship and morphological similarities between our strains and *M.gallisepticum*, information already available on the pathogenesis of this species is considered to be particularly relevant.

The close association between various mycoplasma organisms and epithelial cells of TOCs of several hosts has been observed by a number of workers and this has included *M.gallisepticum* inoculated into chicken embryo TOCs (Abu-Zahr and Butler, 1976, 1978; Hirano *et al.*, 1978; Dykstra *et al.*,1985). Attachment between the organisms and the epithelial surface of the respiratory tract has been accepted as a prerequisite for the cytopathogenicity of certain mycoplasmas including *M.gallisepticum* (Abu-Zahr and Butler, 1976; Uppal and Chu, 1977; Tajima *et al.*, 1979), but the mechanism by which *M.gallisepticum* subsequently causes damage to the host cell is not fully understood.

Interaction of *M.gallisepticum* with chicken or human erythrocytes resulted in haemolysis after prolonged incubation at 37°C (Apostolov and Windsor, 1975) and it was suggested that this was probably due to fusion between the membranes of the organisms and the erythrocytes, followed by entry of the contents of the mycoplasma cell into the erythrocyte.

The release of hydrogen peroxide as a product of cell metabolism (end product of respiration in mycoplasmas) has been considered as a major pathogenic factor produced by the mycoplasma as it causes lysis of erythrocytes *in vitro* (Razin, 1969). It has been found that attachment of *M.pneumoniae* to respiratory tract epithelium provides the opportunity for hydrogen peroxide secreted by the organisms to attack the tissue cell membrane without being rapidly destroyed by catalase or peroxidase enzymes that are present in the extracellular body fluids (Sobeslavsky *et al.*, 1968).

However the production of hydrogen peroxide does not by itself determine the pathogenicity because the non-pathogen *A.laidlawii* produces it, and the swine pathogens *M.hyopneumoniae* and *M.hyosynoviae* produce less hydrogen peroxide than the less pathogenic *M.hyorhinis* and *A.granularum* (Razin, 1978). Cherry and Taylor-Robinson (1971) demonstrated that, although peroxide production did not appear to be important in *M.gallisepticum* pathogenicity for chick embryo TOCs, there was evidence of accumulation of a mildly toxic substance which might have caused tissue damage. Thus the intimate association between adhering mycoplasmas and the host cell surface might provide a means for the transmission of such toxic substances into the host cells and enhance the pathogenicity of the mycoplasmas.

It is possible that the attachment of mycoplasmas is firm enough to protect them from elimination by the clearing action of the ciliated epithelium, and in addition that the attachment provides some nutrients taken from the host cells by the organisms which results in degenerative changes in the cells (Razin, 1978; Tajima *et al.*, 1979).

Reduction of cytadherence of mycoplasmas, resulting in loss of the organism's pathogenicity, has been demonstrated by many workers. For example Lipman and Clyde, (1969) found that a virulent strain of *M.pneumoniae* lost its virulence for hamsters after repeated passage in artificial medium, and had also lost its ability to haemadsorb. Haemadsorption-negative mutants of *M.pneumoniae*, produced by chemical mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, were found to lack pathogenicity in the hamster model system and the mutants were rapidly cleared from the lungs of infected animals (Hansen *et al.*,1981). Similarly, spontaneously occurring haemadsorption-negative mutants of *M.pneumoniae* attached to the respiratory epithelium of hamster tracheal rings *in vitro* at reduced levels and, apart from one mutant class, they all failed to colonize and produce detectable pneumonia in intranasally inoculated hamsters (Krause *et al.*, 1982).

Demonstration of the capability of a mycoplasma to cytadhere does not in itself mean that the organisms should be considered as pathogenic. This suggestion is supported by the occurrence of various mycoplasma species as commensal flora adhering to the epithelial cell surfaces of respiratory and urogenital tracts (Razin, 1985). An example that confirms this is provided by *M.gallisepticum* F strain (a strain used for vaccination of laying hens) which has been shown to be of low virulence but which adhered and colonized the chicken trachea in a similar manner to the pathogenic R strain of the same species (Levisohn *et al.*, 1983).

The association of adherence with the bleb of *M.gallisepticum* was first noticed by Zucker-Franklin *et al.* (1966) who, studying the relationship with leucocytes, found that the bleb was most frequently the contact site. However correlation of the presence of specialised terminal organelles with attachment is not restricted to *M.gallisepticum*. It has been found that *M.pneumoniae* cells also possess such structures at their tip (Biberfeld and Biberfeld, 1970). Terminal organelles similar to

the tip-like organelles of *M.gallisepticum* and *M.pneumoniae* have been found in other mycoplasmas including *M.alvi* (Gourlay *et al,.* 1977), *M.sualvi* (Gourlay *et al.*, 1978) *M.genitalium* (Tully *et al.*, 1983), *M.pirum* (Del Giudice *et al.,* 1985) and *M.mobile* (Kirchhoff *et al.,* 1987), and in the case of *M.genitalium* and *M.mobile* an association with attachment was demonstrated (Tully *et al.*, 1983; Kirchhoff *et al.*, 1987; Stadtlander and Kirchhoff, 1990).

It should be noted that cytadherence is not restricted to those mycoplasma species that possess terminal tip organelles (Razin, 1985). For example, attachment through a close association between *M.bovoculi* and host cells has been noted by Salih and Rosenbusch (1988), and attachment of *M.hyopneumoniae* was also seen through interaction of the organism's membrane with that of host cells (Tajima and Yagihashi, 1982). Furthermore Tajima *et al.* (1982), examining by TEM the tracheas of chickens infected with either a pathogenic or a non-pathogenic strain of *M.gallisepticum*, found a correlation between the presence of a ruthenium red-staining capsule on the organisms and their adhesive properties. The pathogenic organisms appeared to be attached to the microvilli or cilia not only by their terminal bleb but also at other membrane sites through their capsular material. The authors suggested that the capsular material might be an additional means to hold the organisms close to the host cells. Thus it would be of interest to examine strains 4229 and B2/85 for the presence of such capsular material.

With *M.pneumoniae* Hu *et al.* (1977) suggested that a trypsin-sensitive surface protein on the organism, designated P1 was required for membrane-membrane interaction between host and parasite. In further studies it was found that the P1 protein existed at high concentration at the terminal tip organelles of this organism (Baseman *et al.*, 1982; Feldner *et al.*, 1982). However, association of terminal organelles with adherence might not be the only their function since it was suggested that the bleb of *M.gallisepticum* was also associated with movement of the

mycoplasma (Bredt, 1973; Radestock and Bredt, 1977). Furthermore not all the P1 protein of *M.pneumoniae* was clustered at the tip of the organisms but instead numerous less dense P1 regions were present along the mycoplasma surface (Baseman *et al.*, 1982; Brunner *et al.*, 1979).

Other workers produced monoclonal antibodies to P1 protein and used them to inhibit cytadherence (Hu *et al.*, 1982). Feldner *et al.* (1982) produced a monoclonal antibody to P1 protein which inhibited not only haemadsorption but also the gliding motility of the organism. Baseman *et al.* (1982) using a monospecific antiserum to P1 protein found that it inhibited attachment of virulent *M.pneumoniae* to hamster respiratory epithelium, thus confirming the importance of P1 in cytadsorption.

The question of shared antigens existing between species with attachment organelles has also been addressed. Polyclonal and monoclonal antibodies to *M.pneumoniae* protein P1 were non-reactive with whole cell or soluble preparations of *M.genitalium* or *M.gallisepticum* (Baseman *et al.*, 1984) but radio-immunoprecipitation assays performed with hyperimmune rabbit sera raised against each species indicated antigenic cross-reactivity between *M.pneumoniae* and *M.genitalium*.

Hu *et al.* (1984), reported significant serological cross-reactivity between *M.gallisepticum*, *M.pneumoniae* and *M.genitalium* when they used polyclonal antisera to probe the separated proteins of these species by Western blot. Further studies using monospecific and monoclonal antibodies to the P1 protein were carried out by Clyde and Hu (1986) to determine if the cross-reactivity was related to this protein and to see if antigenic determinants were shared by these species. One out of 23 monoclonal antibodies reacted with a protein of *M.genitalium*. Monospecfic antibodies were also found to react with a protein of *M.gallisepticum*, indicating antigenic sharing.

In view of our findings that strains 4229 and B2/85 possess attachment organelles, it would be of interest to see if the antigens common to them and *M.gallisepticum* are associated with the attachment organelles, and also to investigate whether there is any relationship with the tip structures of *M.pneumoniae* and *M.genitalium*. There is indirect evidence one of the proteins shared by 4229 and *M.gallisepticum* (Dupiellet, 1988) could also be the haemagglutinin. This will be discussed in more detail in Chapter 8.

The appearance of strains 4229 and B2/85 and strain S6LP in both chicken and duck tracheal rings changed from a rounded shape when they were away from the epithelial surface to a pear-shape, with their attachment organelles directed towards or attached to the epithelium, when close to the surface. Such observations are in agreement with those of other workers. Abu-Zahr and Butler (1978) found by electron microscopy that *M.gallisepticum* S6 organisms in chick embryo tracheal explants were characteristically cocco-bacilliform except when the organisms were in close contact with the host cells, where they assumed an elongated and irregular form terminating in a bleb that was often embedded in the cell surface. They suggested that the morphological features of the organisms were dependent on the population density and the proximity to the surface of the host cells. Thus individual organisms appeared cocco-bacilliform when the mycoplasmas were in dense aggregates, but where they were well separated they appeared more elongated and irregular.

Similarly, Tajima *et al.* (1979) in their ultrastructural study of chicken tracheal epithelium in birds inoculated with *M.gallisepticum* found that almost all of the organisms that were lying free in the lumen were oval or rounded, whereas they usually assumed an elongated and irregular form when adherent to the epithelial cells through their terminal bleb structure. The bleb was nearly always present in

those organisms that were attached to the epithelial cells and only rarely in those found free in the lumen of the trachea.

It was possible that some of the rounded shapes observed in our study might represent transverse sections of the organisms whereas the pear-shaped structures occurred when they were sectioned through their longitudinal axis. Stadtlander and Kirchhoff (1990) demonstrated that when *M.mobile* was sectioned through its longitudinal axis it showed a characteristic flask-shaped cell form with a head-like structure, whereas other organisms appeared as rounded forms probably due to transverse sectioning.

Attached organisms seemed to push the tip stucture into the epithelial surface of the tracheal lumen although with some organisms a narrow space could be observed between the tip and the host cell surface. A zone of 5 to 7 nm of moderately electron-dense amorphous material and finely fibrillar network was seen by Tajima *et al.* (1979) separating the membrane of *M.gallisepticum* cells from that of chicken tracheal epithelium. This was seen when the unit membranes of the organisms and host cell at the site of contact were cut perpendicularly to their surface, whereas when they were sectioned obliquely the identity of both membranes was lost and appeared as if fusion of the two membranes had occurred or as if the organisms were continuous with the peripheral part of the host cell.

The small spaces may have been due to the presence of mucus between the test organisms and the host cells which might make attachment difficult (Levisohn, personal communication). This possibility was also suggested by Salih and Rosenbusch (1988) in their examination of *M.bovoculi* attached to bovine lung fibroblasts and conjunctival epithelium. They found that a narrow space could be seen between the organisms and the cells. In the conjunctiva the space contained

amorphous strands of extracellular material which the authors suggested could be mucus.

Since the current electron microscopy studies have established that infection of chick and duck embryo TOCs with strains 4229 and B2/85 results in deciliation of the epithelial cells and reveals attachment of the organisms through their tip-like organelles, it is of importance to know if pathogenic changes occur in the in same hosts *in vivo.* Dykstra *et al.* (1985) used the virulent R strain of *M.gallisepticum* in chicken TOC and in live chickens and found a direct correlation between the cytopathological changes seen *in vitro* and *in vivo.* The next Chapter describes some pilot pathogenicity studies carried out in embryos and in live birds.

Chapter 8 Pathogenicity of strains 4229 and B2/85 in embryonated eggs and live birds

Chapter 8 Pathogenicity of strains 4229 and B2/85 in embryonated eggs and live birds

8.1 Introduction

The pathogenicity of a mycoplasma can be influenced by a number of factors which may be related to the organism or to the host. Factors related to the organism include virulence (which varies according to the strain), tropism, numbers of organisms and route of infection, while those related to the host include the species, breed (genetic constitution), age (embryo, neonate or older bird), immune status, intercurrent infections and environmental factors (Jordan, 1979; 1985). Such factors should be taken into consideration when assessing mycoplasma pathogenicity.

Of the 17 currently recognised *Mycoplasma* species of avian origin, only four are established as pathogens causing disease and economic loss in domestic poultry. These are *M.gallisepticum* and *M.synoviae* in chickens and turkeys and *M.meleagridis* and *M.iowae* in turkeys.

M.gallisepticum is the causative agent of chronic respiratory disease (CRD) in chickens and turkeys. It is commonly manifested by tracheitis and airsacculitis, and also by infectious sinusitis in turkeys (Jordan, 1979) and results in economic loss caused by down-grading of carcases of broilers and turkeys. Suboptimal egg production is seen in infected layers and reduced hatchability in chicks and poults. In rare cases the organism has been associated with encephalopathy in turkeys and with arthritis, tenosynovitis and salpingitis in chickens (Jordan, 1990).

M.synoviae is the causative agent of 'infectious synovitis' in chickens and turkeys affecting the joints, bursae and tendon sheaths. It may also be associated with airsacculitis in chickens, and with respiratory distress and infectious sinusitis in

turkeys (Jordan, 1979). The economical importance of the disease is associated with joint lesions, lameness and retarded growth in broiler chickens, replacement pullets and turkeys (Jordan, 1990).

M.meleagridis infection in turkeys affects particularly the young poults causing poor growth, airsacculitis, osteodystrophy, crooked necks and abnormalities of the primary wing feathers. In breeding birds it causes suboptimal hatchability (Jordan, 1990).

Organisms of the *M.iowae* group infect turkeys and chickens and can be recovered from a number of a free-flying birds. *M.iowae* appears to be widespread in domestic turkey flocks and can reduce hatchability (McClenaghan *et al.* 1981) and give rise to poor quality poults (Jordan, 1990). In experimental infections *M.iowae* has been reported to cause lesions in the respiratory tract and the locomotory system of turkeys and chickens (Yoder and Hofstad, 1964; Rhoades, 1981a; Bradbury and McCarthy, 1981; 1984; Bradbury *et al.*, 1988b; Bradbury and Kelly, 1991).

Exacerbation of mycoplasma disease in poultry can occur as a consequence of synergism with other avian pathogens. Examples include *M.gallisepticum* and *M.synoviae* with Newcastle disease (ND) virus, infectious bronchitis (IB) virus and pathogenic strains of *Escherichia coli* in chickens, and *M.gallisepticum* with certain strains of *E.coli* and influenza A virus in turkeys (Jordan, 1979). Synergism between *M.meleagridis* and *M.synoviae* (Rhoades, 1977) and *M.meleagridis* and *M.iowae* (Rhoades, 1981b) has been reported.

On the other hand, amelioration of disease by *M.gallisepticum* has been demonstrated in infection of chickens with a mild strain of NDV (Nonomura and Sato, 1975), and Bradbury (1975) described the protective effect of *M.synoviae* on subsequent infection with *M.gallisepticum* in chickens.

In the preceding Chapters, it has been proposed that strains 4229 and B2/85 represent a new species of *Mycoplasma* and it has been shown that both organisms are pathogenic *in vitro* for chick and duck embryo tracheal organ cultures. However, the pathological importance of these strains and the related strains isolated from geese by Dupiellet *et al.* (1990), has not been fully established.

Strain 4229 presented some evidence of pathogenicity in geese and goose embryos (Buntz, 1987), in chick and duck embryos, and in live ducks and turkeys (Dupiellet , 1988) but Yagihashi *et al.* (1988) found no evidence of pathogenicity of this strain, B2/85 or one of the goose strains in 8 week old SPF chickens.

In view of the above it was considered worthwhile to investigate further the pathogenicity of strains 4229 and B2/85, and to commence with a study *in ovo* using chick and duck embryos.

8.2 Preliminary pathogenicity studies in embryonated chicken and duck eggs

8.2.1 Materials and Methods

Materials:

Embryonated eggs

Fertile chicken and duck eggs were from the sources described in 7.1.2. They were fumigated, washed, stored, and incubated as before.

Mycoplasma strains

Strains 4229, B2/85 and *M.gallisepticum* S6LP and S6HP were used from once-cloned stocks. They had undergone 7 *in vitro* passages in this laboratory when used here, although the previous passage history of 4229 was unknown. Strain S6LP was at passage level 4 and S6HP at 105.

Preparation of inocula

Inocula were prepared and counted as described for tracheal organ cultures (7.1.2) and the cultures were diluted to give approximately 10^7 CFU/ml.

Procedure:

At 7 days of incubation for chicken embryos and 8 days for duck embryos, groups of 10 fertile eggs were inoculated via the yolk sac with 0.1 ml of the appropriate culture containing approximately 10^6 CFU. A control group of 10 eggs (chicken or duck, as

appropriate) was inoculated via the yolk sac with sterile MB without thallium acetate and a further group of 10 uninoculated eggs was incubated alongside.

The broth control group was inoculated first, followed by the infected groups. For the inoculations the eggs were candled and the area of embryo development was marked with pencil. The shell around the blunt end of each egg was disinfected with Merthiolate solution to reduce bacterial contamination from the shell surface, and a hole was punched through the shell in the middle of the area using a disinfected pin projecting approximately 1 mm through a rubber bung. The inocula were introduced by syringe fitted with a 21 gauge 11/2 in needle which was passed down and along the long axis of the egg just beyond the centre. After inoculation the hole in the shell was sealed with molten paraffin wax.

The eggs were returned to the incubation and candled daily for viability. Embryo mortality was recorded and dead embryos were removed for sampling as described below. Deaths within 24 hours of inoculation were regarded as non-specific, since they may have resulted from physical trauma due to the inoculation procedure.

Chicken eggs with viable embryos at 19 days post-inoculation and duck embryos at 25 days were placed in a refrigerator at 4°C to kill the embryos and minimize haemorrhage on opening (Jordan, 1983). These, and also those embryos which had died during the course of the experiment, were opened using aseptic technique, starting with the control embryos. Samples of the extra-embryonic fluid and yolk sac fluid were swabbed onto MA and BA. Yolk samples were spread thinly on MA and, if the embryo was large enough, an oesophageal swab was also taken and inoculated onto MA and BA.

MA plates were incubated in candle jars at 37°C and examined daily for up to 3 weeks for colonies. For both the chicken and duck experiment two selected isolates

of each test strain were tested for identity by indirect immunofluorescence as described in 6.2.2. Inoculated BA plates were incubated aerobically at 37°C and examined at 24 hours.

8.2.2 Results

Mycoplasma inocula

The viable counts of the inocula are given in Table 8:1 The inoculum of strain B2/85 contained lower numbers than the other three strains and none of them contained the intended level of 10^7 CFU/ml.

Mortality of chicken and duck embryos

In embryonated chicken eggs strains 4229 and B2/85 caused 80% and 87.5% mortality respectively, and most of these deaths occurred during the last week of inculbation between 13 and 19 days (Table 8:2; Figure8:1). Most of the mortality following inoculation with strains S6LP and S6HP occurred between 13 and 15 days and both strains gave rise to 100% mortality.

In duck eggs strain 4229 caused a total mortality of 80% and B2/85 of 70% by the end of the experiment at 25 days. Most of these deaths occurred between days 18 and 22 of incubation, being days 10 to 14 post-inoculation (Table 8:2; Figure8:2). Strain S6LP appeared to be more pathogenic than the other 3 strains, causing slightly earlier embryo deaths and resulting in 100% mortality by the end of the experiment. There were no embryo deaths within the first 24 hours of inoculation but during the experiment two broth-inoculated and one uninoculated embryo died.

Table 8:1 Details of mycoplasma strains used in embryo experiments

Strain	CFU/ml	Passage level
4229	5.2×10^6	71
B2/85	2.5×10^5	7
S6LP	7.7×10^6	4
S6HP	5.0×10^6	105

1. No. of *in vitro* passages in our laboratory

Table 8:2 Chicken and duck embryo mortality following yolk sac inoculation

Strain	Chick embryos		Duck embryos	
	No.[1]	%[2]	No.	%
4229	8/10	80	8/10	80
B2/85	7/8[3]	87.5	7/10	70
S6LP	10/10	100	10/10	100
S6HP	10/10	100	7/10	70
Broth	1/8[3]	12.5	2/10	20
Uninoculated	0/10	0	1/10	10

1. Number of dead embryos/number inoculated
2. Percent embryo mortality
3. 2 eggs were discarded due to death within 24 hours of inoculation

Fig.8:1 Mortality of chick embryos

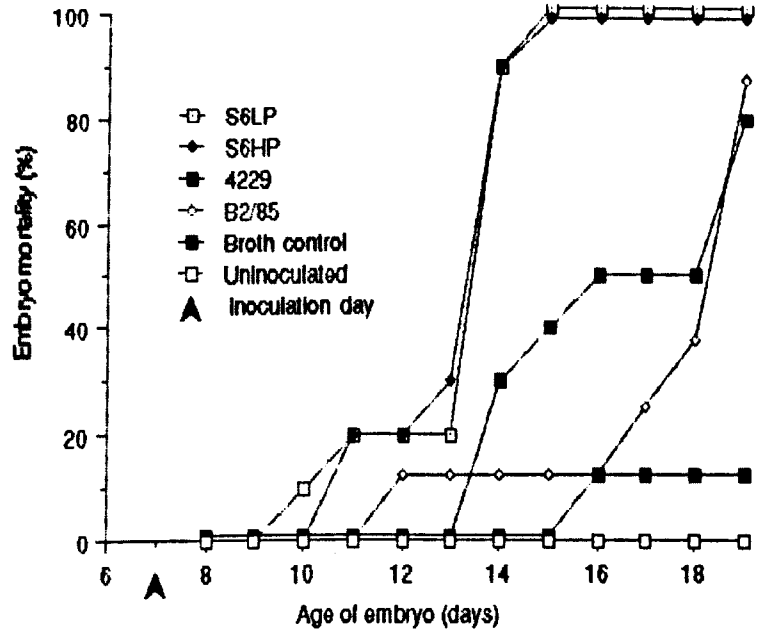
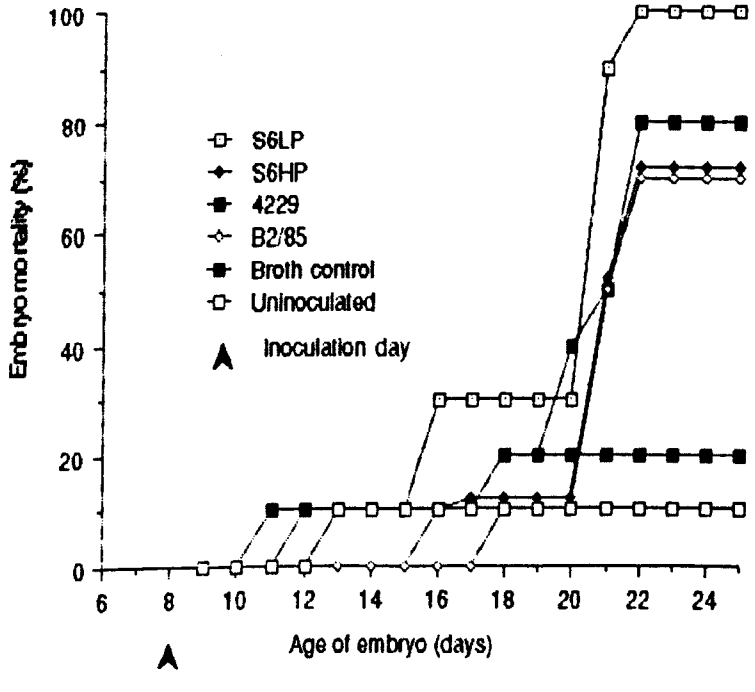


Fig.8:2 Mortality of duck embryos



Recovery and identification of mycoplasmas and bacteria

There was a high rate of reisolation of all mycoplasma strains from all sites sampled in both chicken and duck embryos. All embryos were culturally positive and from most sites (Tables 8:3 and 8:4). Immunofluorescence tests confirmed the identity of the organisms. No mycoplasmas were recovered from control embryos and no bacteria were isolated.

8.2.3. Discussion

These *in ovo* experiments have confirmed the pathogenicity of strains 4229 and B2/85 for both chicken and duck embryos.

Embryonated chicken eggs were used because they have been considered suitable as a method for assessing the pathogenicity of avian mycoplasmas (Yoder and Hofstad, 1964; Power and Jordan, 1973; 1976). However, various factors may influence the results such as the number of organisms inoculated, route of inoculation and the genetic constitution of the host (Power and Jordan, 1973).

Embryonated duck eggs have received little attention in mycoplasma studies (Jordan, 1979), possibly due to difficulties in obtaining eggs from a mycoplasma free source. However, in these experiments, mycoplasmas were never isolated from our control eggs. Furthermore several batches of embryonated duck eggs from the same commercial source have been monitored for mycoplasmas for other experiments and have never yielded an isolate (J M Bradbury, personal communication).

Duck embryos were used because the duck was the original host of strain 4229 and we wished to compare our findings with those of Dupiellet (1988) who had already

Table 8:3 Mycoplasma isolations from chick embryos

	No. and % reisolations from							
	Extraembryonic fluid		Yolk sac		Oesophagus		Total	
Strain	No.	%	No.	%	No.	%	No.	%
4229	10/10[1]	100	10/10	100	10/10	100	30/30	100
B2/85	8/8	100	8/8	100	8/8	100	24/24	100
S6LP	10/10	100	10/10	100	9/10	90	29/30	97
S6HP	10/10	100	10/10	100	10/10	100	30/30	100

1. No. positive /no. sampled

Table 8:4 Mycoplasma isolations from duck embryos

	No. and % reisolations from							
	Extraembryonic fluid		Yolk sac		Oesophagus		Total	
Strain	No.	%	No.	%	No.	%	No.	%
4229	10/10	100	10/10	100	10/10	100	30/30	100
B2/85	8/10	80	10/10	100	10/10	100	28/30	93
S6LP	9/10	90	10/10	100	9/10	90	28/30	93
S6HP	10/10	100	10/10	100	10/10	100	30/30	100

1. No. positive /no. sampled

carried out comparative pathogenicity studies with strain 4229, a goose strain and strain S6 of *M.gallisepticum* in both chicken and duck eggs. The yolk sac route of inoculation was used by Dupiellet and is easily performed at various stages of incubation (Power and Jordan, 1973). A possible disadvantage of this route is that, when attempting to recover the inoculated mycoplasmas directly from the yolk on MA, the lipids may mask the mycoplasma colonies (Jordan, 1979). Streaking the yolk in a thin layer is suggested to avoid this problem (Jordan, 1983) and therefore in this experiment the yolk was spread thinly and presented no difficulty in recognising colony growth.

Inoculation of chicken and duck embryos at 7 and 8 days of incubation respectively was also chosen to repeat the work of Dupiellet (1988). Since duck embryos have a longer incubation period than chicken embryos, this timing was presumably so that his chick and duck embryos were at approximately the same stage of development.

In our experiments, all four mycoplasma strains caused mortality of chicken and duck embryos, and strain S6LP killed a higher percentage of both chicken and duck embryos than strains 4229 and B2/85. These findings support those in the chick and duck TOCs, although lack of correlation has been observed between *in ovo* pathogenicity results and other *in vivo* or *in vitro* methods for evaluating mycoplasma pathogenicity (Levisohn *et al.*1985; 1986).

Our results were not in complete agreement with those of Dupiellet, (1988). Although he also found that *M.gallisepticum* S6 killed chicken embryos earlier and in greater numbers (80%) than strain 4229 (approximately 70%), he observed that strain 4229 was more virulent for duck embryos (92% mortality) than S6 (60-70%). He supported his findings with those in chicken and duck embryo primary fibroblast cell cultures and concluded that host specificity was demonstrated by strain 4229.

There are a number of factors that may have accounted for the differences between our results. One could be the breed of bird, because the duck embryos used in our experiment were of the Pekin breed (*Anas platyrhynchos*) whereas those used by Dupiellet were a Pekin-Barbary cross and are more related to the Muscovy duck (*Cairina moschata*). Our chick embryos were from our own light hybrid SPF birds while those used by Dupiellet were of commercial origin.

Another factor might be the difference in the *in vitro* passage levels of the 4229 cultures used, since the virulence of a *Mycoplasma* species may vary with its passage level (Power and Jordan, 1976). The passage level of strain 4229 used by us was unknown and it is possible that Dupiellet used a very low passage level of this organism.

Strain B2/85 was used at a passage level of 7 in these experiments and was therefore relatively low, but the resulting mortality in both chicken and duck embryos was still less than that with strain S6LP of passage level 4. This might have been because the two species of embryo were less susceptible to the partridge isolate than to S6LP or it may simply have been related to passage level.

M.gallisepticum has been isolated from naturally infected embryonated duck eggs (Bencina *et al.* 1988b) and it has been reported that strain S6 could multiply in experimentally-infected embryonated duck eggs, but to lower titres than in embryonated chicken eggs (Yamada and Matsuo, 1983). These workers found no mortality occurred by 12 days after inoculation of 10^2 CFU into the yolk sac of either 13 day old duck embryos or 10 day old chicken embryos. This is in direct contrast to our results of high mortality in both chicken and duck embryos. However the *in vitro* passage history of the mycoplasma was not mentioned by these authors, also the

embryos were older than ours at the time of inoculation and they received a considerably lower dose of the organisms.

The inoculum dose may be of significance in influencing the outcome of embryo infection. Dupiellet used a dose of 10^2 to 10^3 CFU in contrast to our infecting dose of 10^6 CFU, and it has been found that with certain highly pathogenic strains of *M.gallisepticum*, including low passage S6, there was a relationship between the dose of inoculum and embryo mortality. Whereas with less pathogenic strains there was no strict correlation with dose (Levisohn *et al.*, 1985), strain S6LP caused higher chick embryo mortality than other less pathogenic strains of *M.gallisepticum* using inocula between 10^6-10^7 CFU (Power and Jordan, 1976) but when using inocula of 10^3 or less, the embryo mortality was reduced, even with the virulent S6 (Power and Jordan, 1973).

The fact that strains 4229 and B2/85 caused later death than S6LP could possibly be because continued incubation allowed massive multiplication of the organisms in the yolk, resulting in inhibitory effects on the embryo, as has been demonstrated with less pathogenic strains of *M.gallisepticum* (Levisohn *et al.*, 1985) and with 4 strains of *M.iowae* (Bradbury and McCarthy, 1983). In our study mycoplasmas were reisolated from almost all sites sampled, indicating that inoculation of these organisms by the yolk sac route resulted in extensive proliferation and dissemination.

The duck embryo mortality pattern caused by strains S6LP and S6HP provides evidence for variation of virulence between these two strains, since the percentage was lower with the more highly passaged strain. In chick embryos this difference was not apparent and is in agreement with the report of Power and Jordan (1976) that differences in virulence between S6 of low and high passage were not obvious in chick eggs inoculated via the yolk sac at 7 days of age and incubated at 37°C. In

their study differences were only observed when the inoculated chick embryos were incubated at 34°C.

Strain 4229 has been reported by Buntz (1987) to cause mortality in goose embryos following inoculation at 13 days of incubation via the yolk sac. Thus the pathogenicity of strain 4229 for geese, duck and chicken embryos is well established and the present study confirms that strain B2/85 also causes mortality of chicken and duck embryos.

It would be interesting to investigate the pathogenic mechanisms in more detail and also to see if these organisms are pathogenic for other avian embryos, particularly the partridge. Unfortunately a preliminary survey suggests that UK partridge flocks carry numerous mycoplasma species (J M Bradbury, personal communication) and it may be difficult to locate a source of mycoplasma free eggs.

Following the demonstration of pathogenicity of strains 4229 and B2/85 *in ovo*, preliminary studies on the pathogenicity of strain B2/85 for young chicks and poults were undertaken in young chicks and poults.

8.3 Preliminary pathogenicity studies on strain B2/85in chicks and turkey poults

These investigations were restricted to strain B2/85 because the Importation Licence issued for strain 4229 by the Ministry of Agriculture, Fisheries and Food did not permit its use in live birds.

8.3.1 Materials and Methods

Materials:

Chicks and poults

Chicken eggs were from the Department's SPF flock (7.1.2). They were washed, set and incubated at 37°C in a commercial incubator as before and at 18 days of age the fertile eggs were transferred to hatching trays in the same incubator.

One day old male poults were obtained from a commercial turkey company[1]. The breeding flock was known to be free of infection with *M.gallisepticum*, *M.synoviae* and *M.meleagridis* but its freedom from *M.iowae* infection was not guaranteed. Thus swabs were taken from the oesophagus and cloaca of all poults on arrival for attempted mycoplasma isolation on MA and in MB. Any culturally positive poults would be excluded from the group as early as possible in the experiment.

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1 British United Turkeys Limited, Tarvin, Cheshire, U.K.

Mycoplasma strain

A once-cloned culture of strain B2/85 was used for the initial infection in both experiments at a passage level of 7.

Mycoplasma media

MB and MA were as described previously (2.3).

Chicken and turkey infusion broth (CIB and TIB)

These broths were prepared by the method described by Bradbury (1972). Thus chicken or turkey meat (muscle, liver and heart) was cut in small pieces and 100 ml of distilled water was added to 50 g of meat and allowed to stand in a refrigerator overnight. The infusion was placed in a boiling water bath for 30 minutes and, after cooling, was filtered through Whatman No.1 filter paper. An 80 ml volume of the infusion was supplemented with 0.5 ml sodium chloride, the pH was adjusted to 7.5 and the mixture was autoclaved at 10 lb sq.in for 30 minutes. After cooling the following ingredients (2.2) were added:

Glucose solution (10%)	1.0 ml
Penicillin solution	0.5 ml
Thallium acetate solution	1.0 ml
Phenol red solution	2.0 ml
Heat inactivated chicken or turkey serum	20.0 ml

Preparation of inocula

For the initial inoculation the B2/85 culture was thawed and diluted in MB (without thallium acetate)to provide inoculum containing approximately 10^6 CFU/ml. A viable count was performed at the time of inoculation to confirm the number of CFU.

For intravenous administration the inocula were prepared in CIB or TIB as appropriate. For this the organism was first passaged in the relevant broth to adapt it bringing the passage level in CIB was 14 and in TIB was 15 by the time it was ready for inoculation. The cultures were stored at -60°C and the inoculum was prepared by diluting a thawed aliquot in CIB or TIB without thallium acetate to give approximately 10^6 CFU/ml.

Procedure:

Experiment 1. Infection of chicks

Twenty-six chicks were hatched and divided randomly into two groups and each group was housed in a separate disinfected wooden house. A group of 17 chicks was infected at one day of age via the right thoracic air sac with 0.1 ml of the diluted broth culture containing 1.2 x 10^5 CFU of strain B2/85. A control group consisting of 9 chicks was similarly inoculated but with sterile MB without thallium acetate. All birds were supplied with water and with commercial food *ad libitum* (chick starter crumbs) and the housing and management of both groups were similar.

Chicks were examined daily for clinical signs. At weekly intervals up to six weeks, swabs for mycoplasma culture were taken from the oesophagus and cloaca. All swabs were dipped in MB before use. At 3 weeks and 6 weeks, 5 birds in the infected group and 2 in the control group were killed by intravenous injection of

pentobarbitone [1] and were examined for gross lesions. Samples for mycoplasma isolation were taken from right and left infra-orbital sinuses, oesophagus, trachea, right and left lungs, right and left thoracic air sacs, bursa of Fabricius and cloaca. Blood was also taken from each chick prior to killing and the serum collected after 24 hours.

As no obvious disease was noted and no antibodies were detected, it was decided to use the remaining 7 birds in the infected group to produce chicken antiserum to strain B2/85. Thus, at 13 weeks of age a blood sample was taken from each chick which was then given an intravenous inoculation of 1 ml of CIB culture of strain B2/85 containing 2.3 x 10^6 CFU. The remaining birds in the control group were inoculated intravenously with sterile CIB without thallium acetate. The chicks were kept under observation and were bled at 3, 7 and 14 days post-inoculation at which point they were killed by intravenous injection of pentobarbitone. They were examined for gross lesions but mycoplasma culture was not undertaken.

Experiment 2. Infection of poults

After the initial swabbing on arrival, 36 poults were divided randomly into two experimental groups of 26 and 10 and were housed in two separate disinfected wooden houses. In the first group, 21 poults were infected with 0.1 ml of broth culture of strain B2/85 at one day of age via the right thoracic air sac, and also via the eye with 0.05 ml. The number of viable mycoplasmas in the inoculum was 1.0 X 10^6 CFU/ml. The remaining 5 poults in this group were left as uninoculated contacts. The 10 poults in the second group were inoculated by the same routes but with sterile MB without thallium acetate.

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[1] Animal Care Limited, York, England

The feeding and management of both groups was similar, and was as described for the chicks except that turkey starter crumbs replaced the chick food.

All the clinical observations and sampling procedures up to 6 weeks were also as described for the chick experiment except for the numbers of birds sampled. Here five infected turkeys were sampled at 3 weeks and a further five at 6 weeks. The five contact birds were also sampled at 6 weeks and three control birds were sampled on both occasions. Nasal swabs were taken from two birds showing slight discharge during the tenth week (see below).

At 11 weeks oesophageal swabs were taken from the infected and control birds and the remaining 10 birds in the infected group were inoculated intravenously with a strain B2/85 using the procedure described in Experiment 1 except that the organism was adapted to turkey infusion broth (TIB). Two control birds were similarly inoculated with sterile TIB. Poults were kept under observation and bled at 3, 7 and 14 days post-inoculation. The experiment was terminated as described for the chickens except that mycoplasma culture was undertaken from the oesophagus, turbinates, infraorbital sinuses and cloaca.

Isolation and identification of mycoplasmas

The methods of isolation were as described by Jordan (1983). Swabs taken during life or at post-mortem examination of the birds were streaked over the surface of MA and then placed in MB. Inoculated plates were incubated at 37°C in candle jars for up to 21 days as described previously (2.5). The numbers of mycoplasma colonies were assessed and recorded on an arbitrary scale. Broths were incubated at 37°C and any showing colour change of the phenol red indicator were subcultured onto MA, and all other broths were incubated for seven days.

The identity of all mycoplasma isolates from both experiments was established by the IFA test (6.2.2) using rabbit antiserum to strain B2/85 (6.2.1). In Experiment 2 the isolates were also tested with antiserum to *M.iowae*.

Serological tests

Rapid serum agglutination (RSA) test

This was performed using B2/85 antigen prepared as described by Bradbury and Jordan (1971b). Commercially prepared stained *M.gallisepticum* S6 antigen[1] was also used.

B2/85 RSA antigen preparation

The organism was grown up in one litre of MB and harvested by centrifugation at 2,000 g for 30 minutes at 4°C. The pellet was washed three times in sterile PBS pH 7.0, resuspended in 2 ml of PBS containing 0.25% phenol and mixed well on a vortex mixer to obtain complete suspension. The prepared antigen was stored at 4°C and a small sample was removed for determination of the protein concentration (6.2.1). A standard antigen containing 6 mg/ml protein was prepared. This was selected because analysis had shown it to be the concentration used in the commercial *M.gallisepticum* antigen.

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1 Intervet, Boxmeer, Holland, Lot 1903, Prod. 04-90

Procedure:

All sera collected from chicks and poults were heat inactivated at 56°C for 30 minutes and tested on the same day. The antigens were brought to room temperature before use.

A 0.02 ml drop of antigen was added to 0.02 ml serum onto a clean microscope slide. and the reactants were mixed by gently rocking the slide for 2 minutes for chicken serum and 3 minutes for turkey serum (Jordan and Kulasegaram, 1968). All sera were tested with both antigens and a known negative control was included in each series together with a known positive serum for *M.gallisepticum*. (Positive control avian serum was not available for B2/85). Positive reactions were recorded when aggregates or clumps of organisms were observed within the reaction time.

Haemagglutination inhibition (HI) test

Preparation of antigens

Each strain (4229, B2/85 and S6LP) was grown up in 200 ml of modified MB. This was as described in 2.3 except that the volumes of both swine serum and yeast extract were reduced to 5% (v/v). The broth was incubated at 37°C and, when the indicator colour changed to orange, the culture was harvested by centrifugation at 3,000 g for 1 hour. It was resuspended to 1:100 of the original culture volume using sterile PBS and, if necessary, stored in aliquots at -60°C. Before each HI test was performed, an aliquot of the test antigen was titrated for HA activity. Three different preparations of each of the strains were used, one being used after storage at -60°C, and the others fresh.

Procedure:

Chicken sera collected 7 and 14 days after the intravenous booster injection were tested for antibodies by the haemagglutination inhibition (HI) test using antigens of strains *M.gallisepticum* and B2/85. The standard technique described by Allan and Gough (1974) was used with 4 HA units of antigen and a 0.75% (v/v) suspension of washed chicken red blood cells in PBS. A titre of 1:40 or above is considered positive. A known positive control for B2/85 was not available but a positive control chicken antiserum to S6LP was included.

Enzyme-linked immunosorbent assay (ELISA)

Chicken sera collected 7 and 14 days after the intravenous booster injection were tested for *M.gallisepticum* antibodies by ELISA using a commercially-produced *M.gallisepticum* antibody test kit[1]. All the procedures were carried out according to the manufacturer's instructions.

8.3.3 Results

Experiment 1 Infection of chicks

Clinical observations and post-mortem lesions

The chickens appeared normal throughout. There were no clinical signs and no gross lesions were seen in the infected or control birds at post-mortem examination.

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Isolation and identification of mycoplasmas

Mycoplasmas were not recovered from either the oesophagus or the cloaca of the chicks for the first four weeks post-infection, with the exception of one bird (no. 12) whose oesophageal swabs were culturally positive at one and two weeks but which was negative thereafter (Tables 8:5 and 8:6). At five weeks 6 out of the 12 remaining birds were positive (5 oesophageal and 4 cloacal isolates) but no recoveries were made from the 7 birds remaining at 6 weeks. Recoveries were made with more frequency via broth than via agar (Table 8:7). All the isolates gave strong positive immunofluorescence with antiserum to strain B2/85. No mycoplasmas were isolated from the birds in the broth-inoculated control group and none were isolated from infected or control birds at post-mortem examination.

Serological tests

RSA reactions did not occur with any of the control sera from the broth-inoculated birds using B2/85 or *M.gallisepticum* antigens. Sera from the infected chickens were also negative with these antigens when tested at 3 and 6 weeks post-inoculation, and when tested prior to the booster inoculation and 3 days afterwards. However, at 7 and 14 days after the booster, all 7 sera reacted with B2/85 antigen (Table 8:8). With *M.gallisepticum* antigen 4 of the 7 sera collected at 7 days post-booster and 5 at 14 days reacted.

Seven days after the intravenous inoculation the ELISA test gave a positive response for *M.gallisepticum* antibody in one out of 7 sera and a suspect reaction in another. Sera from these two birds also gave a suspect reaction at 14 days.

Table 8:5 Experiment 1. Mycoplasma isolations by direct plating from live chicks infected at one day old with strain B2/85

	Age of birds (weeks)[1]											
	1		2		3		4		5		6	
Bird no.	Oes[2]	Cl[3]	Oes	Cl	Oes	Cl	Oes	Cl	Oes	Cl	Oes	Cl
1	0[4]	0	0	0								
2	0	0	0	0								
3	0	0	0	0	0	0	0	0	0	0		
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0								
6	0	0	0	0								
7	0	0	0	0	0	0	0	0	0	0		
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0		
11	0	0	0	0	0	0	0	0	0	0	0	0
12	(+)	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0		
14	0	0	0	0	0	0	0	0	0	0		
15	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0								
17	0	0	0	0	0	0	0	0	0	0	0	0

1. Mycoplasmas were not isolated from broth-inoculated control birds
2. Oesophagus
3. Cloaca
4. Colony numbers were arbitrarily assessed as follows:
   4+ = confluent, 3+ = profuse, 2+ = moderate, 1+ = sparse, (+) = less than 10 colonies,
   0 = no isolation

Table 8:6 Experiment 1. Mycoplasma isolations via broth from live chicks infected at one day old with strain B2/85

	Age of birds (weeks)[1]											
	1		2		3		4		5		6	
Bird no.	Oes[2]	Cl[3]	Oes	Cl	Oes	Cl	Oes	Cl	Oes	Cl	Oes	Cl
1	0[4]	0	0	0	0	0						
2	0	0	0	0	0	0						
3	0	0	0	0	0	0	0	0	0	0		
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0						
6	0	0	0	0	0	0						
7	0	0	0	0	0	0	0	0	0	2+		
8	0	0	0	0	0	0	0	0	4+	4+	0	0
9	0	0	0	0	0	0	0	0	2+	0	0	0
10	0	0	0	0	0	0	0	0	4+	0		
11	0	0	0	0	0	0	0	0	0	0	0	0
12	2+	0	2+	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	4+	(+)		
14	0	0	0	0	0	0	0	0	0	0		
15	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0						
17	0	0	0	0	0	0	0	0	4+	4+	0	0

1. Mycoplasmas were not isolated from broth-inoculated control birds
2. Oesophagus
3. Cloaca
4. Colony numbers were arbitrarily assessed as follows:
   4+ = confluent, 3+ = profuse, 2+ = moderate, 1+ = sparse, (+) = less than 10 colonies, 0 = no isolation

Table 8:7 Experiment 1. Total mycoplasma isolations from chicks infected with strain B2/85

Method of isolation	Age of bird (weeks)	No. and % isolations from Oesophagus		Cloaca	
		No.	%	No.	%
Direct plating	1	1/17[1]	5.8	0/17	0
	2	0/17	0	0/17	0
	3	0/12	0	0/12	0
	4	0/12	0	0/12	0
	5	0/12	0	0/12	0
	6	0/7	0	0/7	0
	Total	1/65	1.5	0/65	0
Via broth	1	1/17	5.8	0/17	0
	2	1/17	5.8	0/17	0
	3	0/12	0	0/12	0
	4	0/12	0	0/12	0
	5	5/12	41.6	4/12	39.3
	6	0/7	0	0/7	0
	Total	7/65	10.7	4/65	6.1

1. No. positive/no. sampled

Table 8:8 Experiment 1. RSA and ELISA tests on sera from chicks infected with strain B2/85

Age of birds (weeks)	Nos. of birds examined	Serological test		
		RSA		ELISA
		B2/85	Mg[1]	Mg[2]
3	5	0/5[3]	0/5	NT[4]
6	5	0/5	0/5	NT
Booster dose days post-inoc.				
3	7	0/7	0/7	NT
7	7	7/7	4/7	1/7[5]
14	7	7/7	5/7	0/7[6]

1. Commercial stained *M.gallisepticum* antigen
2. Commercial *M.gallisepticum* ELISA kit
3. No. positive/no. tested
4. Not tested
5. Serum from another bird gave a suspect positive
6. Two sera were suspect positives. These were from the same two birds that were positive and suspect at 7 days

The HI test was not successful because the negative control sera gave a non-specific reaction with B2/85, 4229 and *M.gallisepticum* antigens. The three different batches of these antigens all reacted similarly.

Experiment 2 Infection of poults

Clinical observations and post-mortem lesions

Three birds from the control group and one from the infected group died within the first 24 hours of life and were discounted from the experiment.

No clinical signs were noted in the turkeys for the first 6 weeks and no lesions were seen at post-mortem examination. At approximately 10 weeks some birds in both the infected and control groups developed diarrhoea. Closer examination revealed two turkeys in the infected group with a slight watery oculo/nasal discharge. In both cases these signs were transient and the same birds appeared normal on the following day. Several birds appeared to have dry crusty material around the nares and one exhibited transient swelling of the right infraorbital sinus. No gross lesions were seen when these birds were examined at the end of the experiment.

Isolation and identification of mycoplasmas

No mycoplasmas were isolated from the broth-inoculated control group during the experiment although at the pre-inoculation swabbing on arrival of the day old poults, 2 of these 10 birds were culturally positive, one from the oesophagus and one from the cloaca. These birds were removed from the experiment and the organisms were subsequently identified by immunofluorescence as *M.iowae*.

No mycoplasmas were isolated at the pre-inoculation swabbing of the group to be infected. After inoculation mycoplasmas were isolated from the oesophagus of every bird in the group but never from the cloaca (Tables 8:9-8:11). Twelve of the 20 birds were culturally positive at 1 week and 19 were positive at 2 weeks. Recoveries were far more numerous and growth more prolific when the samples were processed via broth than when they were plated directly onto agar. Mycoplasmas were also isolated from the oesophagus of one of the 5 uninoculated contact control birds at 1 week and from another bird at 3, 4 and 5 weeks post-inoculation.

When the birds were sampled at post-mortem examination at 3 weeks, mycoplasmas were recovered from the oesophagus of all 5 inoculated birds and from the infraorbital sinuses of 2 and the trachea of one (Table 8:12). At the sampling at 6 weeks mycoplasmas were isolated from the oesophagus of 2 of 5 inoculated birds but from no other sites. At this time mycoplasmas were also isolated from the oesophagus of 2 of the contact birds. No isolations were made from the broth-inoculated controls.

Organisms were also isolated from the nasal swab of one of the two birds showing transient nasal discharge but not from any of the oesophageal swabs that were taken at the time of the booster inoculation. At the final sampling mycoplasmas were recovered from the turbinates of three of the10 birds and from the oesophagus of one.

All mycoplasma isolates gave a strongly positive reaction by immunofluorescence with antiserum to strain B2/85 and there was no reaction with antiserum to *M.iowae*.

Table 8:9 Experiment 2. Mycoplasma isolations by direct plating from live poults infected at one day old with strain B2/85

	Age of birds (weeks)[1]											
	1		2		3		4		5		6	
Bird no.	Oes[2]	Cl[3]	Oes	Cl	Oes	Cl	Oes	Cl	Oes	Cl	Oes	Cl
1	0[4]	0	0	0								
2	0	0	1+	0	1+	0	(+)	0	0	0	0	0
3	1+	0	(+)	0								
4	0	0	0	0								
5	2+	0	1+	0								
6	0	0	0	0								
7	1+	0	1+	0	0	0	0	0	1+	0		
8	0	0	0	0	0	0	0	0	0	0	0	0
9	1+	0	1+	0	0	0	0	0	0	0	0	0
10	(+)	0	1+	0	(+)	0	0	0	0	0	0	0
11	0	0	0	0	1+	0	(+)	0	(+)	0		
12	2+	0	(+)	0	0	0	0	0	0	0	0	0
13	2+	0	1+	0	0	0	0	0	0	0		
14	0	0	(+)	0	1+	0	1+	0	1+	0	0	0
15	0	0	(+)	0	(+)	0	(+)	0	(+)	0	0	0
16	0	0	0	0	0	0	(+)	0	(+)	0	0	0
17	2+	0	0	0	0	0	0	0	0	0		
18	2+	0	(+)	0	0	0	0	0	0	0	0	0
19	2+	0	(+)	0	(+)	0	0	0	0	0	0	0
20	(+)	0	(+)	0	0	0	0	0	0	0		
Uninoculated contact group												
1	0	0	0	0	1+	0	0	0	1+	0		
2	0	0	0	0	0	0	0	0	0	0		
3	0	0	0	0	0	0	0	0	0	0		
4	0	0	0	0	0	0	0	0	0	0		
5	0	0	0	0	0	0	0	0	0	0		

1. Mycoplasmas were not isolated from broth-inoculated control birds
2. Oesophagus
3. Cloaca
4. Colony numbers were scored as follows: 4+ = confluent, 3+ = profuse, 2+ = moderate, 1+ = sparse, (+) = less than 10 colonies, 0 = no isolation

Table 8:10 Experiment 2. Mycoplasma isolations via broth from live poults infected at one day old with strain B2/85

	Age of birds (weeks)[1]											
	1		2		3		4		5		6	
Bird no.	Oes[2]	Cl[3]	Oes	Cl	Oes	Cl	Oes	Cl	Oes	Cl	Oes	Cl
1	4+[4]	0	4+	0								
2	4+	0	4+	0	4+	0	(+)	0	(+)	0	0	0
3	0	0	4+	0								
4	4+	0	3+	0								
5	0	0	4+	0								
6	4+	0	4+	0								
7	3+	0	4+	0	(+)	0	0	0	0	0		
8	0	0	0	0	0	0	3+	0	3+	0	3+	0
9	4+	0	4+	0	4+	0	4+	0	0	0	0	0
10	4+	0	3+	0	3+	0	0	0	3+	0	3+	0
11	0	0	2+	0	4+	0	(+)	0	2+	0		
12	0	0	2+	0	0	0	0	0	0	0	0	0
13	3+	0	4+	0	4+	0	0	0	0	0		
14	4+	0	4+	0	4+	0	4+	0	3+	0	0	0
15	0	0	4+	0	4+	0	0	0	3+	0	3+	0
16	4+	0	4+	0	4+	0	2+	0	2+	0	0	0
17	0	0	4+	0	4+	0	4+	0	3+	0		
18	3+	0	4+	0	3+	0	3+	0	0	0	0	0
19	0	0	2+	0	2+	0	2+	0	0	0	0	0
20	4+	0	4+	0	(+)	0	2+	0	0	0		
Uninoculated contact control group												
1	0	0	0	0	1+	0	1+	0	2+	0		
2	4+	0	0	0	0	0	0	0	0	0		
3	0	0	0	0	0	0	0	0	0	0		
4	0	0	0	0	0	0	0	0	0	0		
5	0	0	0	0	0	0	0	0	0	0		

1. Mycoplasmas were not isolated from broth-inoculated control birds
2. Oesophagus
3. Cloaca
4. Colony numbers were scored as follows: 4+ = confluent, 3+ = profuse, 2+ = moderate, 1+ = sparse, (+) = less than 10 colonies, 0 = no isolation

Table 8:11 Experiment 2. Total mycoplasma isolations from live poults infected with B2/85

Method of isolation	Groups	Age of bird (weeks)	No. and % isolations from the oesophagus[1]	
			No.	%
Direct plating	Inoculated	1	11/20[2]	55
		2	13/20	65
		3	6/15	40
		4	5/15	33
		5	5/15	33
		6	0/10	0
		Totals	40/95	42
Via broth		1	19/20	95
		3	13/15	87
		4	10/15	67
		5	8/15	53
		6	3/10	30
		Totals	65/95	68
Direct plating	Contact	1	0/5	0
		2	0/5	0
		3	1/5	20
		4	0/5	0
		5	1/5	20
		Totals	2/25	8
Via broth		1	1/5	20
		2	0/5	0
		3	1/5	20
		4	1/5	20
		5	1/5	20
		Totals	4/25	16

1. There were no isolations from the cloaca
2. No. positive /no. sampled

Table 8:12 Experiment 2. Isolation of mycoplasmas from poults at post-mortem

Method of isolation	Group	Age of bird (weeks)	No. examined	No of isolation from						
				Oes[1]	As	IOS	Lu	Tra	Bu	Cl
Direct plating	Inoc[2]	3	5	3	0	0	0	0	0	0
Via broth				5	0	2	0	1	0	0
Direct plating	Inoc	6	5	1	0	0	0	0	0	0
Via broth				2	0	0	0	0	0	0
Direct plating	Contact	6	5	1	0	0	0	0	0	0
Via broth				2	0	0	0	0	0	0

1. Oes = oesophagus, As = right and left thoracic air sac, IOS = infraorbital sinuses, Lu = right and left lungs, Tra = trachea, Bu = bursa of Fabricius and Cl = cloaca
2. Route of infection: Inoc = inoculated birds, contact = contact birds

Serological tests

All sera gave negative reactions in the RSA test with B2/85 and *M.gallisepticum* antigens at 3 and 6 weeks, and also prior to boosting. After boosting 8 out of the 10 infected turkeys gave a positive reaction with B2/85 by 3 days while all 10 were positive at 7 and 14 days (Table 8:13). Two of these birds were also positive with *M.gallisepticum* antigen at 3 days and three at 7 days, but none were positive at 14 days. Positive reactions were not detected using either antigen with any serum from the control birds.

8.3.4 Discussion

In the present preliminary studies, strain B2/85 did not appear to be significantly pathogenic for the experimentally infected chickens or turkeys. No clinical signs or lesions were seen in the chicks and minimal signs were seen in the poults in this uncomplicated infection, but final conclusions on the pathogenicity of this mycoplasma must await further investigations.

As there are many factors relating to both organism and host which may influence the outcome of an infection, we have attempted to address some of these in our experiments. For example, to avoid attenuation by *in vitro* passage we tried to keep this to a minimum by using a culture of strain B2/85 that had been cloned only once.

The age of the host and route of infection may also be important in determining outcome. In general birds in the first few days of life are most susceptible to mycoplasma infections (Jordan, 1979; 1990) and we therefore infected our birds at one day of age in an attempt to maximise any pathogenic effects.

Table 8:13 Experiment 2. RSA tests on sera from poults infected with strain B2/85

Age of birds (weeks)	Nos. of birds examined	RSA test	
		B2/85	Mg[1]
3	5	0/5[2]	0/5
6	5	0/5	0/5
Booster dose days post-inoc.			
3	10	8/10	2/10
7	10	10/10	3/10
14	10	10/10	0/10

1. Commercial stained *M.gallisepticum* antigen
2 No. positive/no. examined

The air sac route of inoculation was used for the chicks, and both airsac and the ocular route for turkeys. Inoculation via the air sac was chosen to see if this mycoplasma was similar to *M.gallisepticum* in causing airsacculitis. Yoder and Hofstad (1964) inoculated broth cultures of avian mycoplasma isolates representing almost all serotypes into the trachea and left thoracic air sac of young chickens and found that airsacculitis was produced only by isolates of *M.gallisepticum*. In turkeys aged between 4 and 16 weeks moderate to severe airsacculitis was produced by isolates of *M.gallisepticum* following inoculation via the sinus, air sac and hock joint. Less extensive airsacculitis was produced by *M.iowae* and moderate airsacculitis produced by *M.meleagridis*. However it was found that inoculation of either chickens or turkeys with *M.gallisepticum* via eye drop, intranasal or intratracheal routes usually resulted in fewer and milder lesions than intrasinus or intra-air sac inoculation (Yoder, 1991).

Airsacculitis was also produced by inoculation of day-old ducklings with *M.gallisepticum* S6LP or *M.anatis* via the air sac (Amin and Jordan, 1978) although no clinical signs were seen.

The influence of different routes of inoculation on the pathogenicity of *M.gallisepticum* has been investigated by Levisohn *et al.* (1986) who found that the low passage F strain did not exhibit pathogenic effects in chickens after intratracheal inoculation at 2 weeks of age but was pathogenic if inoculated by aerosol. Lin and Kleven (1982) found that inoculation of two week old turkeys by eye drop with *M.gallisepticum* strain F (mild) or R (virulent) resulted in minimal air sac lesions, whereas a higher incidence and severity of such lesions was produced with both strains when birds exposed by aerosol.

The decision to give our turkeys an inoculation by eyedrop in addition to the air sac route was made partly because of the absence of clinical signs and lesions in the

chick experiment, but also because infection of turkeys with *M.gallisepticum* tends to cause upper respiratory tract disease, including sinusitis (Jordan, 1979; Yoder, 1991). A further reason was that strain 4229 had been reported by Dupiellet (1988) to cause upper respiratory disease, including conjuncitivitis, in commercial turkeys. However it might have been useful to investigate the pathogenicity of strain B2/85 using the aerosol route rather than the routes that we chose.

The only clinical signs seen in our birds were in the turkey poults, and these were mild and transient consisting of two birds with slight watery oculo/nasal discharge, a few cases of crusty nostrils, and one turkey with transient swelling of the right infraorbital sinus. Some diarrhoea was seen in the control group as well as in the infected group at approximately 10 weeks but this was a problem that was frequently encountered by the breeders in their turkeys at this age. A limited investigation revealed no coccidial oocysts in the faeces but large numbers of rotavirus particles. It was not known if the virus was of any significance but, in view of the occurrence of virus and diarrhoea in both control and infected groups, it was concluded that the mycoplasma was not implicated.

In the commercial turkeys that Dupiellet (1988) inoculated intranasally with strain 4229, clinical disease developed similar to that caused by *M.gallisepticum*. There was severe purulent sinusitis resulting in impaired vision and respiratory rales. Conjunctivitis was seen in all 13 infected birds and airsacculitis in six of them. The infection status of these turkeys was not known but mycoplasmas were not recovered from any of his control birds. However the possibility that a bacterial or viral agent could have exacerbated the effects of the mycoplasma could not be ruled out.

Buntz (1987) noted retarded growth but no severe clinical signs when strain 4229 was inoculated into the thoracic air sacs of one day old goslings, which were then reared for 17 weeks. In another experiment, infection with this strain just prior to

force feeding resulted in smaller livers when the birds were slaughtered. When geese were infected during the laying season, reduced numbers of fertile eggs were obtained. However the disease status of these birds was undefined and they were not from mycoplasma-free stock, although the parents were treated with antimicrobials in advance of the experiments.

Dupiellet (1988) infected 6 week old 'mule' ducks with strain 4229. They were inoculated intranasally and into the infra-orbital sinus, and some also by the intra-peritoneal route. Transient conjunctival irritation and some respiratory signs (rales and sneezing) were noted in all infected birds over the first two weeks, although the author commented that these signs were seen only after careful examination of individual birds. Again the ducks used were of undefined disease status and were not derived from mycoplasma free stock but from parents that had been treated with antimicrobials, moreover none of the birds in the experiment were housed in isolation.

Clinical signs and mortality were not observed by Yagihashi *et al.* (1988) when they inoculated 8 week old specific pathogen free chickens intratracheally with strains 4229, B2/85 or a goose strain (30902). The total passage level in artificial medium for strain 4229 was unknown but for B2/85 and 30902 was 11. The age at infection may have influenced the outcome but the same age of chickens inoculated with two recently isolated field strains of *M.gallisepticum* exhibited respiratory signs.

The absence of significant clinical signs and of gross lesions in experimentally infected birds does not necessarily confirm that the mycoplasma is non-pathogenic since it is known that not all strains of recognised pathogenic mycoplasma species such as *M.gallisepticum* give rise to clinical signs and lesions (Truscott *et al.*, 1974; Yoder, 1986). The pathogenic potential of an organism may not be realised unless

precipitated by some secondary factor such as environmental stress or concurrent infection.

This is well recognised with *M.gallisepticum* and *M.synoviae* (Jordan, 1979) and may well apply to other avian *Mycoplasma* species. For example it was found that when 3 week old chickens were inoculated via the air sac or by aerosol with apparently non-pathogenic *M.gallinarum*, airsac lesions developed if the birds were simultaneously exposed to a combined live NDV and IBV vaccine, or a field strain of IB (Kleven *et al.* 1978). Similarly, it is possible that strain B2/85 could produce disease by acting in synergism with other organisms such as the respiratory viruses or pathogenic strains of *E.coli.*

It would therefore be useful to carry out further investigations with strain B2/85, perhaps administered by aerosol, but also in dual infection with other respiratory pathogens. It would also be necessary to carry out histopathological examination of some of the infected tissues, particularly from the upper respiratory tract of any turkeys with mild respiratory signs, to see if any microscopic lesions are present.

Although no signs or gross lesions were observed in our chickens it was evident that infection had been established in some of the 17 birds since the organism was reisolated from the oesophagus of six and the cloaca of four. In the turkeys 19 out of 20 inoculated birds were culturally positive in the oesophagus by two weeks, and spread to a contact bird was detected as early as one week. This could have occurred by direct contact or by infected airborne droplets in a similar manner to that demonstrated for *M.gallisepticum* (Jordan, 1979).

Despite recovery from the cloaca of the chickens, strain B2/85 was never isolated from this site in turkeys even though the infection appeared to establish more readily and persisted longer in the turkeys.

Recovery of mycoplasma from the cloaca seems to occur with some *Mycoplasma* species and not others (Jordan, 1979). Isolation of *M.gallisepticum* from the cloaca of hatched chicks has been reported following inoculation of yolk sac of 18 day old embryonated eggs (Varley and Jordan, 1978a) but recoveries from this site were only few compared with other tissues, and the organism did not persist. The same workers (Varley and Jordan, 1978b) also reported sporadic recoveries of *M.gallisepticum* from the cloaca of poults following inoculation into the abdomen near the umbilicus at one day old.

Amin and Jordan (1979) also reported recoveries of *M.gallisepticum* from cloaca following inoculation of 3-week-old chickens. MacOwan *et al.* (1983) inoculated day old chicks with a cloacal isolate of *M.gallisepticum* and recovered it from the cloaca of most birds, whether infected intranasally or via the cloaca. He also isolated from the cloaca of contact birds.

Dupiellet (1988) in his experimental infection of six week old 'mule' ducks with strain 4229 reported recoveries of the organism from the cloaca of 10% of birds inoculated intranasally and into the infra-orbital sinus. However organisms were reisolated with greater frequency from other tissues including the trachea.

In our experiment there were many more isolations of strain B2/85 from the turkeys than the chickens. This may have been because they initially received a greater dose than the chickens and were inoculated by two routes. On the other hand, it may be an indication that turkeys are more susceptible to infection with this mycoplasma than chickens. The greater susceptibilty of turkeys to *M.gallisepticum* is well recognised (Yoder, 1991). Varley and Jordan (1978a; 1978b) also found that turkey poults were more susceptible than chicks to three strains of *M.gallisepticum*, with clinical signs and air sacculitis being more prevalent in the former.

Isolation of mycoplasmas from infected chickens and turkeys in the absence of clinical signs and gross lesions is in agreement with other workers. In *M.gallisepticum* infections of chicks and turkeys Varley and Jordan (1978a; 1978b) found that it was possible to isolate in the absence of clinical signs and gross lesions and, conversely, organisms were not always isolated in the presence of signs and lesions.

Our findings for mycoplasma recoveries indicated that initial incubation of the specimens in mycoplasma broth resulted in considerably more isolations than direct plating onto agar. This was true for both the chicken and turkey material. However, Varley and Jordan (1978b) found with *M.gallisepticum* that tissues or swabs cultured directly onto MA gave more recoveries than if they were first inoculated into MB. Furthermore Amin and Jordan (1978) confirmed that recovery of several of the avian mycoplasma species, including *M.gallisepticum*, was favoured by direct plating rather than broth inoculation. Variation in the most suitable method for isolation of different mycoplasma species has been reported, for example it has been found that broth medium is particularly unsuitable for *M.meleagridis* since most isolates do not adapt readily to it (Yamamoto, 1972).

In both chickens and turkeys there was failure to detect antibodies by the RSA test to strain B2/85 after the initial infection. This may have been because the test is not sensitive enough, but another possibility is that there was no antibody response. Lack of response may have been due to immunological immaturity of these young birds, or the mycoplasmas might simply not stimulate a humoral antibody response. Lack of detectable antibodies was also reported by Yagihashi *et al.* (1988) in their RSA tests on chickens inoculated intratracheally at 8 weeks of age with strains B2/85 or 4229 although one out of 10 chickens infected with the goose strain (30902) gave a positive reaction as did 10 out of 10 of those infected with *M.gallisepticum* strains.

It was not stated which antigen was used but, as the study was performed at the time when all these strains were still thought to be *M.gallisepticum*, it was almost certainly an *M.gallisepticum* antigen.

Low levels of RSA reactors have been observed in chicken flocks with natural *M.gallisepticum* infection. Truscott *et al.* (1974) reported isolation of a strain of low virulence from a flock which remained free of clinical symptoms and which showed only small numbers of RSA reactors. Yoder (1986) studied the problem of low levels or lack of antibody response since 1970 in chicken flocks known to be infected with *M.gallisepticum* and concluded that atypical isolates have low pathogenicity and low ability to spread. In addition, they are of less effective in stimulating high rates and levels of RSA and HI antibody reactions.

The intravenous booster inoculations that we gave to the chickens and turkeys resulted in positive RSA responses with B2/85 antigen in all the birds. The turkeys responded more rapidly than the chicks with 8 out of 10 being positive after 3 days, while none of the chickens were positive until 7 days. Some of the birds (5 out of 7 chickens and 3 out of 10 turkeys) also gave an RSA reaction with commercially produced *M.gallisepticum* stained antigen and one chicken was positive at 7 days with the commercial *M.gallisepticum* ELISA kit. Other studies being carried out concurrently in our laboratory using this kit with specific antiserum to *M.gallisepticum* have shown that it is less sensitive than RSA and HI tests. Thus more cross-reactions might have been revealed in our experiment with a more sensitive ELISA.

The serological cross-reactions are not surprising in view of the significant relationship already demonstrated between these organisms by us and by other workers (Yogev *et al.* 1989; Dupiellet, 1988; Dupiellet *et al.* 1990), but it could be argued that they were not specific. For example, it has been observed that certain serum proteins can adsorb from the growth medium onto *M.gallisepticum* during their

growth for production of antigen (Bradbury and Jordan, 1972). When this antigen is inoculated into birds, antibodies to the adsorbed medium components may be produced and can give rise to non-specific RSA reactions. Therefore for the intravenous booster inoculations we took the precaution of growing the antigens in chicken or turkey infusion medium so that the cross-reactions seen between the chicken and turkey antisera and *M.gallisepticum* were unlikely to be due to this phenomenon. Moreover control birds inoculated intravenously with the infusion medium did not give any reactions in RSA or ELISA tests.

It should be emphasised that the RSA reactions with *M.gallisepticum* were only observed in the birds after intravenous inoculation and not when they were infected via the respiratory tract. Further work is therefore required to examine sera from birds naturally infected with this mycoplasma. If naturally infected chickens and turkeys were to give cross-reactions it could have serious implications in interpretation of routine serological monitoring for *M.gallisepticum*.

Unfortunately time did not permit us to perfect the haemagglutination inhibition test in order to further clarify the cross-reactions. It is usually used as a confirmatory test for RSA (Yoder, 1991; Jordan, 1979; 1990). Three different antigen preparations were tested, but all the control sera gave non-specific HI reactions and could not be distinguished from the sera of the infected birds. It would therefore be useful to examine the sera by HI test after treatment with receptor destroying enzyme (neuraminidase) (Roberts *et al.* 1967; Windsor *et al.* 1975) and it would also be of value to investigate the use of fresh broth cultures of strains B2/85 and S6LP as antigen. It was found by Roberts *et al.* (1967) that non-specific reactions in the HI test did not occur when live broth antigens of *M.gallisepticum* were used instead of antigens concentrated by centrifugation. The authors explained that a neuraminidase-like enzyme appeared to be present in broth cultures of *M.gallisepticum* but it was inactive or absent in the 'prepared' antigen possibly due to

the effect of PBS which is known to be toxic for mycoplasmas (Butler and Knight, 1960). Another possibility was that the neuraminidase-like enzyme was extracellular as in certain clostridia, and was therefore eliminated from the prepared antigen during its preparation.

The serological cross-reactions of strain 4229 with *M.gallisepticum* were investigated further by Dupiellet (1988) using immunoblotting. He found two proteins (MW 66 and 60.3 KDa) which were common to *M.gallisepticum* S6, 4229 and three goose strains. It was considered that these might represent the haemagglutinin because Tourtellotte and Forsyth (1988) reported that the haemagglutinin of *M.gallisepticum* was a 64 KDa membrane protein. Antiserum prepared in rabbits to the 64 KDa protein was found to inhibit the haemagglutination of chicken red cells by *M.gallisepticum* (Forsyth *et al.*, 1990). Bradley *et al.* (1988) identified a 69 KDa polypeptide which they suggested might represent part or all of the haemagglutin since a lectin-purified fraction of *M.gallisepticum* containing this polypeptide haemagglutinated chicken red blood cells.

It is also of note that Avakian and Kleven (1990a), using immunoblotting, reported the identification of six immunogenic species-specific proteins of *M.gallisepticum*, one having a molecular weight of 63-65 KDa. In further studies the authors purified the immunogenic proteins, including this one, and evaluated them as potential ELISA antigens (Avakian and Kleven, 1990b). The 63-65 KDa protein detected antibodies 3 days after the RSA and 7 days before the HI test and it was thought that it might represent the haemagglutinin.

Thus it seems possible that the proteins which are common to the duck and goose strains and *M.gallisepticum* are involved in haemagglutination and, if so, this might cause further complications in interpretation of HI tests in the field. The fact that the

HI test tends to be strain specific (Kleven *et al.*, 1988b) could be an advantage in this respect.

In conclusion, our limited experimental studies *in vivo* have failed to demonstrate pathogenicity of strain B2/85 in chickens and have demonstrated only transient, mild respiratory signs in turkeys. From the foregoing discussion it is clear that further investigation is required before definite conclusions can be reached regarding pathogenicity of this mycoplasma in the two hosts. Furthermore, investigation of its pathogenicity for partridges, geese and ducks would be of particular interest since these were the hosts of origin of the strains. It would be useful to undertake studies with strain 4229 as well as B2/85 since it was the former strain that appeared to be pathogenic in the studies in France.

The serological cross-reaction with *M.gallisepticum* in some of the sera of inoculated chickens and turkeys suggests that there might be a practical problem differentiating the infections in the field by conventional serological tests. This aspect requires further investigation.

Once the pathogenicity of the organism is more fully investigated, it may be necessary to undertake work on treatment and control. *In vitro* antibiotic sensitivity tests were carried out by Dupiellet (1988) using strain 4229 and three goose strains with 14 antibiotics and showed that the minimal inhibitory concentrations were of a similar order to those of *M.gallisepticum* S6 and PG31. This provides yet another example of the similarities between the proposed new species and *M.gallisepticum*.

Chapter 9 Preliminary investigations on screening for haemadsorption-negative mutants of *M.gallisepticum*

Chapter 9 Preliminary investigations on screening for haemadsorption-negative mutants of *M.gallisepticum*

9.1 Introduction

The pathogenic mycoplasmas adhere to the epithelial cells of respiratory or urogenital tracts but rarely invade tissue and blood (Razin *et al.*, 1981) and the ability to adhere tightly to the target tissues is considered to be a prerequisite for colonization and development of the disease (Razin, 1985). In spite of demonstration of the adherence of a significant number of human and animal mycoplasma species to a variety of eukaryotic cells (Razin, 1978; 1985), most of the available information has been derived from studies on *M.pneumoniae*, *M.gallisepticum* and *M.pulmonis* (Razin, 1986).

Studies of adherence phenomena *in vitro* have been performed by various investigators and in several ways. They include interactions with erythrocytes, adsorption to tissue culture cells, adsorption to organ cultures, the use of monoclonal antibodies, analysis of attachment proteins and electron microscopy studies. Some of these have already been discussed in Chapter 7.

Interactions between erythrocytes and mycoplasmas have been observed *in vitro* in HAd, HA and haemolysis tests. In HAd erythrocytes adsorb to mycoplasma colonies, in contrast to HA where the organisms in suspension adhere to and agglutinate the erythrocytes. The HAd process is often studied as a model for the adherence of mycoplasma to respiratory epithelium (Razin, 1985) despite the fact that erythrocytes are not the natural target cells of these organisms.

Most investigations on *M.pneumoniae* support the usefulness of erythrocytes as models in attachment studies. It has been found that HAd to colonies by virulent

strains of *M.pneumoniae* (Manchee and Taylor-Robinson, 1968) bears certain similarities to attachment to respiratory epithelium, since the receptors for both contain sialic acid residues, and trypsin pretreatment of the organisms eliminates attachment to both cells types (Sobeslavsky *et al.*, 1968). Similarly, the major receptors for *M.gallisepticum* are host membrane sialoglycoconjugates (Banai *et al.*, 1978; Razin, 1985)

The above observations on *M.pneumoniae* prompted workers to isolate spontaneous or induced mutants from this species that were deficient in haemadsorption. Such mutants were used for further evaluation of the relationship between HAd and attachment to respiratory epithelium and to permit molecular comparisons between wild-type and mutant mycoplasmas in an attempt to gain better understanding of the pathogenicity of these organisms.

A large number of mutagenic agents are known but most mycoplasma studies have involved the use of the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG), and Hansen *et al.* (1979) successfully isolated two haemadsorption negative (HAd^-) mutants from *M.pneumoniae* in each of two independent experiments using this mutagen. The mutants had a reduced ability to attach to hamster tracheal rings *in vitro* when compared with the wild-type parent *M.pneumoniae* strain.

Feldner and Bredt (1983) also derived HAd^- mutants of *M.pneumoniae* using NTG. They classified them into two groups according to their polypeptide patterns and found that the proteins of one group were identical to that of the wild-type strain, whereas those in the second group differed from the wild-type strain by the absence of one or more polypeptides. The ability of all these mutants to grow and attach to a glass surface was either weak or completely absent, and they varied in their capacity to adsorb erythrocytes of different animal species, suggesting that adherence was mediated by different binding mechanisms in the different species.

HAd$^-$ mutants have not only been derived by the use of chemical mutagens. Other investigators (Krause *et al.*, 1982) have isolated mutants which are spontaneously deficient in HAd properties. The protein profiles, attachment and virulence capabilities of 22 such mutants were investigated and the protein profiles allowed grouping into four classes. All attached at reduced levels to the respiratory epithelium of hamster tracheal rings *in vitro* and none of them was capable of causing pneumonia in intranasally inoculated hamsters. Apart from one mutant class, all failed to colonize. Some avirulent HAd$^-$ mutants were found to lack the attachment protein P1.

Other studies relating to the P1 protein of *M.pneumoniae* and its role as a major adhesion protein were discussed in Chapter 7 (Hu *et al.*, 1977; Hu *et al.*, 1982; Baseman *et al.*, 1982; Feldner *et al.*, 1982; Krause and Baseman, 1982). However it should be noted that P1 protein has been detected in HAd$^-$ populations of virulent *M.pneumoniae* (Kahane *et al.*, 1985).

Thus, with *M.pneumoniae*, isolation of derived and spontaneous HAd$^-$ mutants has permitted characterization by protein profiles, attachment capabilities and virulence. Following the initial work on HAd$^-$ *M.pneumiae* mutants, the entire structural gene of P1 protein has been determined, cloned and sequenced (Su *et al.* 1987).

NTG treatment has not only been used to derive HAd$^-$ mutants, it has also been used to produce temperature-sensitive (Ts) mutants of several *Mycoplasma* species with a view to using them for immunisation of the respiratory tract. These include *M.pneumoniae* (Steinberg *et al.*, 1969), *M.synoviae* (Nonomura and Imada, 1982), *M.gallisepticum* (Lam *et al.*, 1984; Whithear *et al.*, 1990) and *M.pulmonis* (Wayne *et al.*, 1990).

The work described in this Chapter was a preliminary attempt to isolate HAd$^-$ mutants from *M.gallisepticum* by different approaches. These were by screening for spontaneously HAd$^-$ mutants, and by mutagenesis using ultraviolet light and NTG. Attempts to isolate HAd$^-$ mutants of *M.gallisepticum* have not so far been reported with any of the above approaches, and the reason for chosing to work with this species rather than with strain 4229 or B2/85 was because of its recognised importance as a pathogen.

As with *M.pneumoniae*, HAd$^-$ mutants should be useful in providing further understanding of the adherence mechanisms of *M.gallisepticum* and in elucidation of the pathogenesis of this organism. If the techniques are successful with *M.gallisepticum*, they could then be applied to strains 4229 and B2/85.

9.2 Materials and Methods

Hepes suspension medium

Hepes [1]	0.59 g
Sodium chloride	1.46 g
Glucose	0.18 g

The above mixture was dissolved in distilled water and made up to 250 ml. It was sterilized by autoclaving at 15 1b/sq.in for 15 minutes and stored at 4°C.

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1 BDH Chemicals Ltd., Poole, Dorset, U.K.

Phosphate buffered saline (PBS) PH 6.7 & 7.2

These were prepared as described in 2.4 and the pH was adjusted with either 1M sodium hydroxide or 1M hydrochloric acid as appropriate. The buffers were autoclaved at 15 lbs/sq.in for 15 minutes.

N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

(1-methyl-3-nitro-1-nitrosoguanidine)

For safety reasons the NTG was obtained preweighed in glass ampoules[1] and placed inside a self-contained plastic glove bag[1]. All subsequent manoevres were carried out within the bag inside a fume cupboard. The NTG was dissolved in PBS, pH 6.7 and then further diluted in PBS to produce solutions containing the various required concentrations as indicated in the different experiments below.

***Mycoplasma gallisepticum* strains**

The strains of *M.gallisepticum* used are given in Table 9:1. Each strain had been cloned three times by filtration (2.6), grown up in bulk and stored in aliquots of 30 ml at -60°C. After at least 24 hours, an aliquot of each strain was thawed, a viable count was carried out and the identity of the organism was confirmed by IFA test (6.2.2).

Haemadsorption test (HAd)

The method of Gardella and DelGiudice (1983) as described in 5.2.12 was used for screening for colonies of HAd^- mutants.

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1 Sigma Chemical Company, St. Louis, MO, U.S.A.

Table 9:1 ***M.gallisepticum*** **strains examined for spontaneous and UV-induced HAd⁻ mutants**

Organisms	Passage level
S6LP	4
S6HP	104
B14/85[1]	≈ 12
A514[2]	unknown
PG31[2]	unknown

1. Isolated from the eye of a chicken in a flock with reduced egg production
2. Highly attenuated

Experiment 1 Screening for spontaneously-occurring and UV-induced HAd⁻ mutants

This experiment was designed to screen for colonies of spontaneous HAd⁻ mutants in five strains of *M.gallisepticum* (Table 8:1) and also to attempt to derive HAd⁻ mutants from these strains by exposure to UV light. The method used was based that of Miller (1972) but it was necessary to select a suitable diluent in which to suspend the organisms for treatment. Two suspension media had been investigated in an earlier mutagenesis experiment with *M.gallisepticum* (Chaudhry, 1985) and Hepes buffer appeared to be satisfactory. It was decided to confirm this finding.

Examination of Hepes buffer as a suspension medium

A 30 ml aliquot of culture was thawed and centrifuged at 4,000 g for 30 minutes. The pellet was re-suspended in 7.5 ml of Hepes buffer. A viable count was carried out immediately (time=0) then the suspension was placed in an ice bath for 5 minutes to prevent further growth. It was then removed and a further viable count was carried out (T=5). The suspension was kept at room temperature and viable counts were conducted after 30 (T=30) and once again 60 (T=60) minutes.

Killing of *M.gallisepticum* strains by different exposure times to UV light

Before the mutagenesis experiment proper, a suitable UV exposure dose had to be determined for each strain since a high level of kill (99%) is recommended.

A UV lamp[1] emitting at 254 nm was placed at a height of 30 cm above the bench and allowed to warm up for a minimum of 30 minutes. Meanwhile an aliquot of the

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1 Baird and Tatlock Ltd., Essex, U.K.

appropriate culture was thawed, centrifuged, re-suspended to its original volume in Hepes buffer and placed in an ice bath for 5 minutes as described above. The mycoplasma suspension was dispensed in 4 ml volumes in six 9 cm sterile glass Petri dishes and five were exposed to UV light for varying times with the lid removed. The sixth dish represented zero time. The dish was rocked gently to and fro in order to keep the contents moving. Using a stop watch, the exposure time (30 seconds, 1, 2, 5, 10 or 15 minutes) was measured and immediately on completion the lid was replaced and the Petri dish put into a light-proof black polythene bag for 5 minutes to prevent light repair of the DNA. A viable count was carried out on each culture on removal from the bag and the percentage of organisms killed by each exposure was determined.

Preliminary screening of *M.gallisepticum* strains for spontaneously-occurring and UV-induced HAd$^-$ mutants.

A 30 ml aliquot of culture of each strain was thawed, centrifuged, resuspended in Hepes buffer and placed in an ice bath as described above. A 0.1 ml volume of the suspension was removed into 0.9 ml MB and then serial tenfold dilutions were made up to 10^{-6} and a viable count was carried out. To search for spontaneously occurring mutants a volume of 1 ml was removed from each of the 10^{-4}, 10^{-5} and 10^{-6} dilutions, and spread onto each of 3 separate MA plates using a bent glass rod.

For UV treatment a 4 ml volume of the culture suspension in Hepes was placed in a Petri dish and exposed to UV light for the length of time previously established to achieve a 99% percent kill of the strain under study. Tenfold dilutions of UV-treated organisms were then plated out for a viable count and an extra MA plate for each of the 10^{-1} to 10^{-3} dilutions was also set up to screen for HAd$^-$ colonies. Plates were incubated at 37°C until the colonies were of suitable size to perform the HAd test. Each candidate HAd$^-$ colony was picked off the agar and inoculated into 1 ml MB.

As soon as colour change occurred, the broth was frozen at -60 °C for later retesting by HAd test. The number of colonies screened was calculated from the viable counts.

Experiment 2 Screening for NTG-induced HAd⁻ mutants

Pilot tests to establish the percentage kill of NTG-treated organisms

As with the UV experiment, pilot tests had to be carried out to establish the amount of NTG necessary to give a high percentage kill with each strain of *M.gallisepticum*.

Overnight broth cultures of *M.gallisepticum* strains S6LP, S6HP and A514 were harvested by centrifugation at 12,000 g at 4°C for 20 minutes (Rottem, 1983) and pellets harvested from 50 ml of broth were resuspended in 10 ml of NTG solution containing 25, 50 or 100 µg/ml. A control pellet was resuspended in 10 ml PBS.

After incubation for 30 minutes at room temperature the organisms were again harvested by centrifugation at 12,000 g for 20 minutes. The pellets were then washed three times in PBS with harvesting as above after each wash. They were then resuspended in 5 ml MB and a viable count was carried out on each sample, including the control, to establish the percentage kill at the three different concentrations of NTG.

This procedure was repeated using NTG concentrations of 100, 200 and 300 µg/ml.

Preliminary screening of *M.gallisepticum* S6LP treated with a dose of 300 µg/ml NTG for NTG-induced HAd⁻ mutants

A concentration of 300 µg/ml was chosen as a result of the pilot tests and the method described above was applied to *M.gallisepticum* S6LP. Screening for mutants was as described after UV exposure except that the treated pellet was resuspended in 5 ml MB before dilutions were made. Due to inconsistent results, the experiment was repeated using incubation times of 90 and 120 minutes.

9.3 Results

Experiment 1 Screening for spontaneously-occurring and UV-induced HAd⁻ mutants

Examination of Hepes buffer as a suspension medium

The effect of Hepes buffer on the six strains of *M.gallisepticum* is shown in Figure 9:1. All strains lost some viability but it was considered from these results that Hepes buffer was a suitable diluent for them.

Killing of *M.gallisepticum* strains by different exposure times to UV light

The percentage kill of *M.gallisepticum* strains with duration of UV light exposure is given in Figure 9:2. In all strains there was a large loss of viability with increasing exposure time. With S6LP the percentage kill after two minutes was more than 95% and was 100% by five minutes. The other strains were somewhat more resistant. The exposure times selected for the subsequent experiment were as follows: S6LP = 2 minutes; S6HP = 5 minutes; A514 = 10 minutes; B14/85 = 10 minutes; PG31= 15 minutes.

Fig. 9:1 The effect of Hepes buffer on viability of *M.gallisepticum* strains

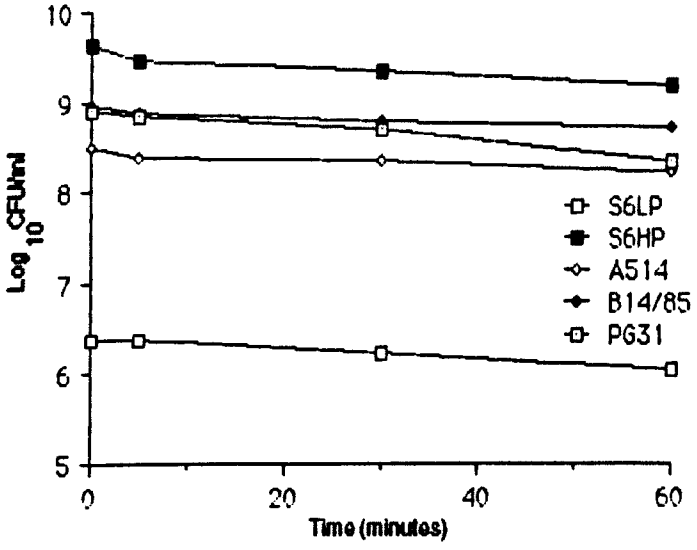
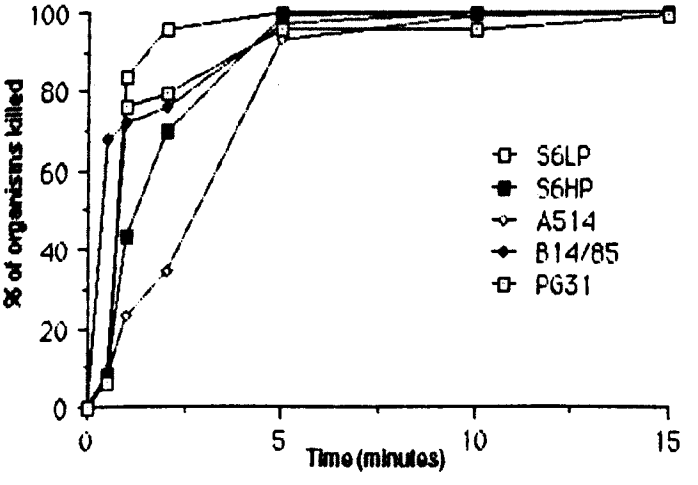


Fig. 9:2 Percentage kill of *M.gallisepticum* strains exposed to UV light



Preliminary screening of *M.gallisepticum* strains for spontaneously-occurring and UV-induced HAd⁻ mutants.

The percentage kill of all the strains with the selected exposure times was approximately 99%. The numbers of colonies screened for spontaneous and UV-induced HAd⁻ mutants are shown in Table 9:2. In all 124 suspect HAd⁻ colonies were subcultured and retested for HAd but no candidate mutants were isolated. A considerable practical problem was encountered during the screening of colonies by the HAd test in that large numbers of them became detached from the agar surface either on addition of the erythrocytes or at the later washing stages.

Experiment 2 Screening for NTG-induced HAd⁻ mutants

Pilot tests to establish the percentage kill of NTG-treated organisms

The percentage kill of the three *M.gallisepticum* strains is shown in Figures 9:3 and 9:4. Strain S6, whether high or low passage, was somewhat resistant to treatment for 30 minutes with NTG at concentrations of 25, 50 and 100 µg/ml in the first pilot test although strain A514 was more susceptible. Increasing the dose of NTG to 200 or 300 µg/ml increased the percentage kill for S6 to over 90% but the results for A514 were not consistent with those of the first test. Therefore it was decided to use only strain S6LP, treated with a dose of 300 µg/ml NTG, for preliminary screening for HAd⁻ mutants.

Table 9:2 Examination of *M.gallisepticum* strains for spontaneous and UV-induced HAd⁻ mutants

Strain	No. of colonies screened for spontaneous HAd⁻ properties		No. of colonies screened for UV-induced HAd⁻ properties	
S6LP	8,191	(9)[1]	712	(9)
S6HP	35,298	(12)	1,015	(14)
A514	17,094	(10)	2,653	(34)
B14/85	232	(9)	852	(9)
PG31	8,158	(9)	769	(9)
Total no. of colonies screened	68,973	(49)	6,001	(75)

1. No. of suspect HAd⁻ colonies subcultured for retesting

Fig. 9:3 Percentage kill of *M.gallisepticum* strains treated with NTG

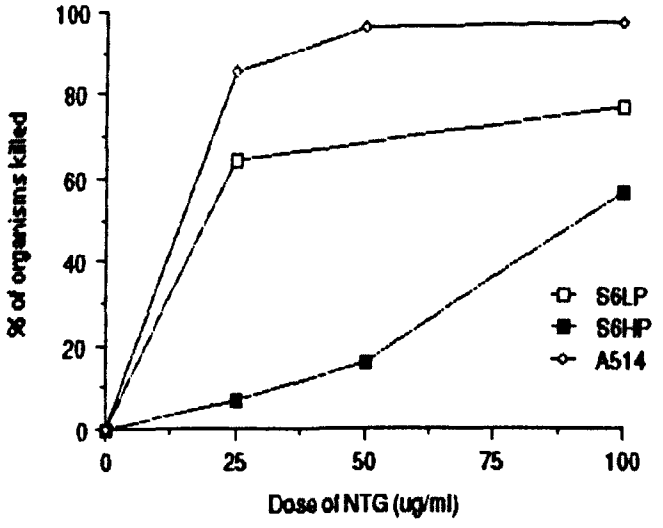
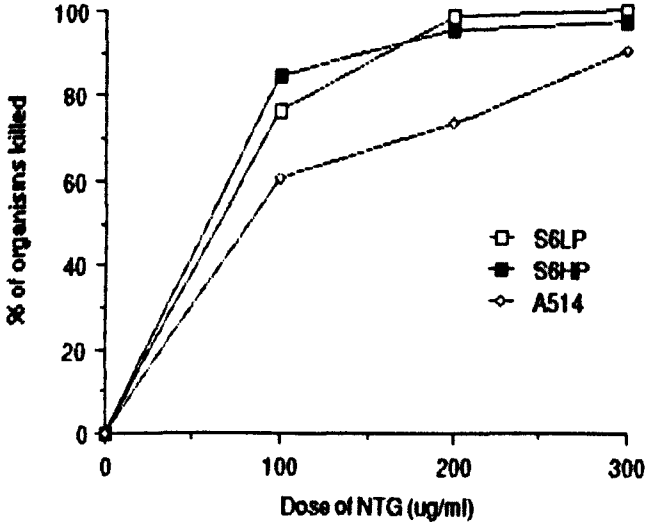


Fig. 9:4 Percentage kill of *M.gallisepticum* strains treated with NTG



Preliminary screening of *M.gallisepticum* S6LP for NTG-induced HAd⁻ mutants treated with dose of 300 µg/ml NTG.

The percentage kill from this treatment was considerably lower than anticipated, being only 58%. From a total of approximately 3,000 colonies screened by HAd 36 suspect HAd⁻ colonies were picked and regrown for confirmatory testing. All gave a positive reaction. A 99.9% kill was finally achieved by incubation with 300 µg/ml NTG for 120 minutes. Some 14,000 colonies were subsequently screened by HAd but no HAd⁻ mutants were found. During the HAd tests, there was again a considerable problem with detachment and floating of most of the mycoplasma colonies.

Discussion

This study was performed in similar ways to those successfully applied to the human pathogen *M.pneumoniae*. Unfortunately no candidate mutant colony was isolated in our experiments out of many thousands screened. These findings appeared to be due to technical difficulties associated with the HAd test because a large proportion of the mycoplasma colonies detached from the agar at the stage of addition of the chicken erythrocyte suspension, and there was further detachment when the suspension was poured off after incubation, and during the subsequent washing procedure. The colony 'ghosts' that were left behind sometimes resembled HAd⁻ colonies, but more confusing were the colonies that floated on the surface of the liquid and apparently did not come into contact with the erythrocytes. Some of these colonies seemed to be redeposited onto the agar at the end of the procedure, and then appeared to be HAd⁻.

Such a phenomenon does not appear to have been encountered by other workers who successfully isolated HAd⁻ mutants of *M.pneumoniae* (Hansen *et al.*, 1979;

Krause *et al.*, 1982; Feldner and Bredt, 1983). However detachment of *M.gallisepticum* colonies has frequently been encountered in this laboratory during the washing procedures involved in the indirect immunofluorescence test. Relatively few *Mycoplasma* species seem to present this problem and it is not known why it is seen with *M.gallisepticum* in particular. Later in this discussion, attempts that were made to avoid this phenomenon are outlined.

For preliminary screening for spontaneous and UV-induced HAd$^-$ mutants, Hepes buffer was selected as a suspension medium. The buffer has been used by other workers to keep the organisms intact (Rottem *et al.*, 1981).

Magnesium sulphate (0.1 M) has been used as a diluent for *E.coli* UV mutagenesis (Miller, 1972) in order to avoid loss in viability due to the production of toxic products resulting from the irradiation of complex media, but Chaudhry (1985) found that this compound was more toxic than Hepes buffer for *M.gallisepticum*.

A high level of kill (99-99.9%) is recommended for UV mutagenesis and it is necessary to establish a killing curve before undertaking mutagenesis experiments (Miller, 1972). *M.gallisepticum* required several minutes of exposure to UV light to achieve high levels of kill.

The selection of spontaneous and UV-induced HAd$^-$ mutant colonies from different strains of *M.gallisepticum* proved unsuccessful although this was probably more due to the method of screening than to the method chosen to induce mutagenesis. Nevertheless it was decided to attempt to induce chemical mutagenesis using NTG.

As with UV mutagenesis it is necessary with NTG to obtain a high percentage kill (95%-99%) in the exposed organisms in order to increase the likelihood of inducing mutation in the surviving population (Hansen *et al.*, 1979). As the chemical is such a

potent mutagen, it was hoped that even if most colonies were lost during HAd screening, there would be a greater chance of finding a mutant in the remaining colonies than after UV treatment. From our pilot experiments, it was observed that exposure to a dose of 300 µg/ml of NTG for 30 minutes should be suitable for strain S6LP since it gave a 99.9% kill of the organism. This is considerably more than was used by Hansen *et al.* (1979) with *M.pneumoniae* who found that a dose of 25 µg/ml for 30 minutes induced 95% mortality.

However the required percentage kill was not achieved in the experiment proper, and we could only obtain a 99% kill by incubating the mycoplasma with NTG for 120 minutes. Even then we were not successful in isolating potential NTG-induced *M.gallisepticum* mutants and this was thought to be due to the repeated difficulties encountered in screening for HAd^- colonies because of their detachment from the agar.

Subsequent experiments were carried out with the aim of avoiding the phenomenon of detaching colonies in the HAd test. Factors investigated included: the age of colonies (incubation period), the degree of crowding of colonies, the type of gel used in the medium (conventional agar or agarose), their concentration and pH, drying of the agar using agents such as silica gel and phosphorus pentoxide, and the effect of increasing the specific gravity of the diluent used in the HAd test by addition of glycerol. However none of these investigations resulted in significant reduction in the numbers of detaching colonies.

A method for screening for HAd^- colonies that might avoid detachment of colonies was to grow them on membranes placed on the MA surface. BioTrace NT[1] membrane (nitrocellulose) and BioTraceRP[1] membrane (charged-modified nylon 66)

1 Gelman Sciences, Michigan, U.S.A.

and Millipore[1] membranes were used. Colonies grew well on all these membranes but they still detached on addition of diluents.

In further attempts to avoid this problem, a number of other approaches to selecting out possible cytadsorption negative mutants were investigated at the suggestion of Dr J B Baseman (personal communication). These included screening for non-haemolytic colonies, separation with Percoll, and an enrichment technique. Since they were not included in the foregoing descriptions of experiments they will be described very briefly here.

Attempted selection of *M.gallisepticum* mutants by the haemolysis technique was carried out by two methods. In the first, inoculated MA plates were overlaid with agarose containing chicken erythrocytes and incubated for 1-4 days at 37°C, whereas in the second method the grown colonies were overlaid with agarose containing the chicken erythrocytes, and then incubated. Neither technique proved to be reliable because the haemolysis reaction was not consistent.

Attempts were made to separate an enriched population of HAd$^-$ mutants using Percoll[2]. The technique was based on the idea that, after adding erythrocytes to a suspension of *M.gallisepticum* containing potential HAd$^-$ mutants, the HAd positive organisms would attach to the cells leaving the mutants unattached. The suspension is then centrifuged on a Percoll gradient in an attempt to separate the two populations. After this, the contents of the upper and lower gradient layers are plated onto MA and the colonies tested for HAd. In our hands the Percoll did not appear to be toxic to the mycoplasmas but no potential mutants were isolated by this method.

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1 Millipore Corporation, Watford, U.K..

2 Sigma,Chemical Company, St.Louis, MO, U.S.A

Another method for selection of spontaneous HAd$^-$ mutants colonies, known as the selective enrichment technique was described by Leith *et al* (1983) who used it to isolate a HAd$^+$ revertant of an HAd$^-$ mutant of *M.pneumoniae* (Hansen *et al.*, 1979). This method was attempted here with *M.gallisepticum* but again without success. A total of 187 suspect HAd$^-$ colonies were derived by this method but on retesting they were positive for HAd.

We have thus experienced considerable difficulties either in inducing or in isolating HAd$^-$ mutants from *M.gallisepticum*, although these findings do not indicate that the organisms are not undergoing mutation. It has been noted that this *Mycoplasma* species has a high level of spontaneous mutation (J D Pollack, personal communication via J M Bradbury) and it is likely therefore that HAd$^-$ mutants were present in our experiments, but that the technical difficulties prevented us from finding them. Other workers have successfully isolated Ts mutants of *M.galliisepticum* after treatment with NTG (Lam *et al.*, 1984; Whithear *et al.*, 1990) and NTG has the advantage of being a more powerful mutagen than UV light (Miller, 1972). Lam *et al.* (1984) exposed the mycoplasma to 50 or 100 µg/ml NTG for 15 minutes while Whithear *et al.* (1990) reported using a dose of 100 mg/ml for 30 minutes.

A method of screening *M.gallisepticum* that might be a possible approach in future is described by Shimizu and Nagatomo (1989). In this technique, called the adhesion-haemadsorption test, the mycoplasmas are attached to the surface of plastic U-shaped microtitre plates and the erythrocytes are added to the wells. The authors found that the test was positive for all twelve tested strains of *M.gallisepticum* but negative with the other *Mycoplasma* and *Acholeplasma* species. A possible way to screen for mutants would be to grow colonies on agar as before but, instead of adding erythrocytes to the plate, the individual colonies could be picked off and each cultured in a separate microtitre well. After growth has occurred each well would be

screened by the adhesion-haemadsorption test, but this process would be very laborious since large numbers of colonies would need to be tested.

Several thousand colonies were screened in our various experiments. Krause *et al.* (1982) isolated 22 spontaneously occurring HAd^- mutants of *M.pneumoniae* out of total 10,000 colonies screened while only two HAd^- mutants were isolated from each of two independent chemical mutagenesis experiments using NTG as mutagen (Hansen *et al.*, 1979).

The current studies were considered only as a preliminary attempt to isolate HAd^- mutants of *M.gallisepticum*, but it is obvious that the technical difficulties must be resolved before this line of work can be pursued further.

Chapter 10 General conclusions and suggestions for future work

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10.1 General conclusions

In the work presented in this thesis, we have undertaken investigations on two similar untyped avian mycoplasma strains, 4229 and B2/85, which were isolated from duck and partridge respectively, and which were both originally identified as *M.gallisepticum*. Further molecular and serological studies on strain 4229 had shown it to be somewhat different to *M.gallisepticum*, and DNA:DNA hybridization demonstrated only approximately 40% homology between them. This led to the suggestion that strain 4229 and related strains might represent a new avian *Mycoplasma* species.

Restriction endonuclease analysis of the DNA of strains 4229 and B2/85 using three enzymes revealed no differencies between them and both strains were used in our investigations.

It was concluded from our taxonomic studies that the organisms were members of the class *Mollicutes*. There was no evidence of a cell wall and the cells were surrounded by a triple-layered membrane. An attachment organelle was clearly seen in both strains. The colony appearance and the morphology and size of the cells corresponded to those reported for *Mollicutes*. There was no reversion to bacterial forms on culture in the absence of inhibitors. There were no helical forms and the organisms were not obligate anaerobes. These last two properties respectively showed that they were not members of the family *Spiroplasmataceae* , or of the order *Anaeroplasmatales* .

Tests to demonstrate a growth response to sterol were not conclusive but the organisms were sensitive to digitonin and sodium polyanethol sulphonate, which

gave an indirect indication of their sterol requirement and showed that they did not belong to order *Acholeplasmatales*. Thus these strains could be assigned to the order *Mycoplasmatales*, family *Mycoplasmataceae*. Since they did not hydrolyse urea, the organisms could not be placed in the genus *Ureaplasma* and were assigned to the genus *Mycoplasma*.

Biochemical characterization provided a description of strains 4229 and B2/85 and showed that they had the same biochemical profile as *M.gallisepticum*: ie. glucose fermentation but no hydroysis of arginine, no evidence of phosphatase, no film and spots production and no liquefaction of serum. There was reduction of tetrazolium chloride aerobically and anaerobically and the organisms were HA and HAd positive. Earlier evaluation of the G+C contents of the DNA of strain 4229 and three related strains from geese gave a mean value of 31.8 mol% which was lower by 1.6 mol% than the value obtained for *M.gallisepticum* (Dupiellet, 1988; Dupiellet *et al.*, 1990).

Serological examination by GI and immunofluorescence tests showed that strains 4229 and B2/85 are distinct from the recognised avian and mammalian *Mycoplasma* species with the exception of *M.gallisepticum*. Distinction from this species could only be made on the basis of the DNA:DNA hybridization results of Dupiellet *et al.* (1990).

The above findings are considered to fulfil the requirements of the Subcommittee on the Taxonomy of *Mollicutes* (1979) for proposing a new species. Since this organism is so similar to *M.gallisepticum*, we intend to propose for it the name *Mycoplasma imitans*, and to nominate 4229 as the type strain. The specific epithet is derived from the Latin verb in the gerund form meaning "imitating".

Pathogenicity studies carried out in France with strain 4229 indicated its pathogenicity for goose, duck and chicken embryos and and that it gave rise to clinical signs in ducks and turkeys. The pathogenicity of strain B2/85 was not known.

We have carried out pathogenicity studies of 4229 and B2/85 in chick and duck embryo tracheal organ cultures and in embryonated chicken and duck eggs. The two strains were pathogenic for both chick and duck TOCs causing ciliostasis. They appeared to be less virulent than strains S6LP and S6HP of *M.gallisepticum* that were used for comparative purposes and which generally produced slightly earlier ciliostasis. Growth of the organisms occurred only in the presence of tracheal sections and the ciliostasis was independent of the presence of 5% calf serum in the medium, although addition of serum was found to encourage the growth of the organisms resulting in more rapid ciliostasis.

Immunofluorescent staining of infected TOCs enabled location of strains 4229, B2/85 and *M.gallisepticum* S6LP on the tracheal epithelium of chicken and duck tissues. Examination by transmission electron microscopy revealed a close association between strains 4229 and B2/85 and the epithelial cell surface of both chicken and duck TOCs. The organisms nearest to the surface were often attached through their tip-like organelles in a similar way to the attachment of *M.gallisepticum* S6LP through its bleb organelle. Thus the tip-like organelles seen earlier in the cell pellets of strains 4229 and B2/85 appear to have a role in cytadherence. Similar structures have been found in certain other *Mycoplasma* species and they are thought to be important in pathogenesis.

We established also that strains 4229 and B2/85 caused mortality of chicken and duck embryos thus confirming an earlier report by Dupiellet (1988). Our findings indicated that strains 4229 and B2/85 are less pathogenic than *M.gallisepticum* S6LP *in ovo*.

In experimental infections of one day old chickens and turkey poults strain B2/85 showed little evidence of pathogenicity. There were no clinical signs in the chickens and only transient and very mild signs in the turkeys. No gross lesions were seen but the organism was recovered from the respiratory tract of infected birds, especially the turkeys. The organism appeared to persist longer in the turkeys than in the chickens and spread to contact turkeys was demonstrated within a week.

Sera from the infected chickens and turkeys were negative at 3 and 6 weeks post inoculation when tested by RSA with both B2/85 and *M.gallisepticum* antigens. After an intravenous booster inoculation homologous RSA reactions were detected in all birds. In addition, five out of seven chickens and three out of 10 turkeys gave agglutination in the *M.gallisepticum* RSA test. The chicken sera were also tested using an *M.gallisepticum* ELISA kit and one showed a positive result and one a suspect positive. Thus, even if the organism proves to be non-pathogenic, infection of chickens and turkeys might cause practical problems during routine screening for *M.gallisepticum* due to the serological relationship.

Final conclusions on pathogenicity must await further experimental studies. This mycoplasma, being very similar to *M.gallisepticum*, may require the presence of other factors such as environmental stress or concurrent viral or bacterial infection before showing evidence of pathogenicity.

Chapter 9 of this thesis describes preliminary attempts to isolate haemadsorption negative (HAd^-) mutants of *M.gallisepticum* in order to investigate attachment mechanisms by the approach used by other workers with *M.pneumoniae*. Cultures were examined for spontaneous occurrence of HAd^- colonies and also after treatment with UV light or N-methyl-N'-nitro-N-nitrosoguanidine. Several thousand

colonies were screened but due to technical difficulties no mutants were isolated. Modifications of the screening method were unsuccessful.

9.2 Suggestions for future work

During these studies a number of aspects warranting further investigation were apparent and the most important of these are mentioned briefly below:

1. Further investigations of the sterol requirements for strains 4229 and B2/85. The basic methods for assessing direct growth response to sterol require investigation since even the positive control organisms did not give the expected result in our hands.

2. Several new *Mycoplasma* species have been described since the completion of the serological studies and these species need to be cross-tested with strains 4229 and B2/85.

3. Elucidation of the mechanisms of pathogenicity of strains 4229 and B2/85 in tracheal organ culture and study of the pathogenicity in TOCs and embryos of other hosts such as the partridge, goose and turkey.

4. Further studies on the importance of the tip-like organelles in strain 4229 and B2/85 to see if they have a function other than attachment. It would also be interesting to see if there is any antigenic relationship between the attachment organelle and that of *M.gallisepticum*. Comparison of antigens with *M.pneumoniae*, *M.genitalium* and the other mycoplasmas with tip structures would also be of value.

5. Since *M.gallisepticum* possesses capsular material that may be related to pathogenicity it would be interesting to examine strain 4229 and B2/85 for similar material using ruthenium red staining.

6. Experimental infections with strain B2/85 and 4229 in chicken and turkeys of different ages and by different routes of inoculation. The pathogenicity should also be investigated in the goose, duck and partridge if mycoplasma free stock can be obtained.

7. Further experimental studies in chickens and turkeys to investigate possible synergism of strain B2/85 with other avian pathogens such infectious bronchitis virus, Newcastle disease virus or pathogenic strains of *E.coli*.

8. Since strain 4229 and *M.gallisepticum* apparently share major antigens, which may include haemagglutinin, it is important to establish whether this is likely to cause any complications in the interpretation of serological tests in the field. In these studies the HI test was unsatisfactory and needs to be perfected before undertaking further experimental work in both chickens and turkeys.

9. The epidemiology of the proposed new species in domestic poultry and game birds needs to be established.

REFERENCES

Abu-Zahr, M.N. and M.Butler. (1976). Growth, cytopathogenicity and morphology of *Mycoplasma gallisepticum* and *M.gallinarum* in tracheal explants. J.Comp.Path., 86: 455-463.

Abu-Zahr, M.N. and M.Butler. (1978). Ultrastructural features of *Mycoplasma gallisepticum* in tracheal explants under transmission and stereoscan electron microscopy. Res.Vet.Sci., 24: 248-253.

***Ad Hoc* Committee of the International Committee for Systematic Bacteriology.** (1987). Report of the *Ad Hoc* Committee on reconciliation of approaches to Bacterial Systematics. Int.J.Syst.Bact., 37: 463-464.

Adler, H.E., R.Yamamoto and J.Berg. (1957). Strain differences of pleuropneumonia-like organisms of avian origin. Avian Dis., 1: 19-27.

Al-Aubaidi, J.M. and J.Fabricant. (1971). The practical application of immunofluorescence (agar block technic) for the identification of mycoplasma. Cornell Vet., 61: 519-542.

Allan, W.H. and R.E.Gough. (1974). A standard haemagglutination inhibition test for Newcastle disease (1) A comparison of macro and micro methods. Vet. Rec., 95: 120-123.

Allen, T.C., J.O.Stevens, E.R.Florance and R.O.Hampton. (1970). Ultrastructure of *Mycoplasma gallisepticum* isolate 1056. J.Ultrastruct.Res., 33: 318-331.

Aluotto, B.B., R.G.Wittler, C.O.Williams and J.E.Faber. (1970). Standardized bacteriologic techniques for the characterization of *Mycoplasma* species. Int.J.Syst.Bact., 20: 35- 58.

Amikam, D., S.Razin and G.Glaser. (1982). Ribosomal RNA genes in mycoplasma. Nucl.Acids Res., 10:4215-4222.

Amin, M.M. (1977). Avian Mycoplasma: Studies on isolation, infection and control. Ph.D. Thesis, University of Liverpool.

Amin, M.M. and F.T.W.Jordan. (1978). Experimental infection of ducklings with *Mycoplasma gallisepticum* and *Mycoplasma anatis.* Res.Vet.Sci., 25: 86-88.

Amin, M.M. and F.T.W.Jordan. (1979). Infection of the chicken with a virulent or avirulent strain of *Mycoplasma gallisepticum* alone and together with Newcastle disease virus or *E.coli* or both. Vet.Microb., 4: 35-45.

Apostolov, K. and G.D.Windsor. (1975). The interaction of *Mycoplasma gallisepticum* with erythrocytes. I. Morphology. Microbios., 13: 205-215.

Araake, M. (1982). Comparison of ciliostasis by mycoplasmas in mouse and chicken tracheal organ cultures. Microbiol.Immunol., 26: 1-14.

Avakian, A.P. and S.H.Kleven. (1990a). The humoral immune response of chickens to *Mycoplasma gallisepticum* and *Mycoplasma synoviae* studied by immunoblotting. Vet.Microbiol., 24: 155-169.

Avakian, A.P. and S.H.Kleven. (1990b). Evaluation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis purified proteins of *Mycoplasma gallisepticum and M.synoviae* as antigens in a dot-enzyme-linked immunosorbent assay. Avian Dis., 34: 575-584.

Aycardi, E.R., D.P.Anderson and R.P.Hanson. (1971) Classification of avian mycoplasmas by gel-diffusion and growth inhibition tests. Avian Dis., 15: 434-447.

Banai, M., I.Kahane, S.Razin and E.Bredt. (1978). Adherence of *Mycoplasma gallisepticum* to human erythrocytes. Infect.Immun., 21: 365 372.

Bang, F.B. and J.S.F.Niven. (1958). A study of infection in organised tissue cultures. Brit.J.Exp.Path., 39: 317-322.

Barber, T.L. and J.Fabricant. (1971). A suggested reclassification of avian mycoplasma serotypes. Avian Dis., 15: 125-138.

Barile, M.F. and M.W.Grabowski. (1978). Mycoplasma-cell culture-virus interactions: A brief review. In: Mycoplasma Infection of Cell Cultures, Editors: G.J.McGarrity, D.G.Murphy and W.W.Nicols. Plenum, New York. pp.135-149.

Barski, G., F.Cornefert and R.Wallace. (1959). Response of ciliated epithelia of different histological origin to virus infections *in vitro*. Proc. Soc. Exp. Biol. Med., 100: 407-411.

Baseman, J.B. and A.M.Collier. (1974). Diphtheria pathogenesis in guinea pig tracheal organ culture. Infect. Immun., 10: 1146-1151.

Baseman, J.B., R.M.Cole, D.C.Krause and D.K.Leith. (1982). Molecular basis for cytadsorption of *Mycoplasma pneumoniae*. J.Bact., 151: 1514-1522.

Baseman, J.B., D.L.Drouillard, D.K.Leith and J.G.Tully. (1984). Absence of *Mycoplasma pneumoniae* cytadsorption protein P1 in *Mycoplasma genitalium* and *Mycoplasma gallisepticum*. Infect. Immun., 43: 1103-1105.

Bemis, D.A. and J.R.Kennedy. (1981). An improved system for studying the effect of *Bordetella bronchiseptica* on the ciliary activity of canine tracheal epithelial cells. J.Infect.Dis., 144: 349-357.

Bencina, D., D.Dorrer. and T.Tadina. (1987). *Mycoplasma* species isolated from six avian species. Avian Pathol., 16: 653-664.

Bencina, D., T.Tadina and D.Dorrer. (1988a). Natural infection of geese with *Mycoplasma gallisepticum* and *Mycoplasma synoviae* and egg transmission of the mycoplasmas. Avian Path., 17: 925-928.

Bencina, D., T.Tadina and D. Dorrer. (1988b). Natural infection of ducks with *Mycoplasma synoviae* and *Mycoplasma gallisepticum* and mycoplasma egg transmission. Avian Path., 17: 441-449.

Biberfeld, G. and P.Biberfeld. (1970). Ultrastructural features of *Mycoplasma pneumoniae* . J.Bact., 102: 855-861.

Black, F.T. (1973). Modifications of the growth inhibition test and its application to human T-mycoplasmas. Appl.Micrbiol., 25: 528-533.

Boatman, E.S. (1979). Morphology and ultrastructure of the *Mycoplasmatales* . In: The Mycoplasmas, Vol.I. Editors: M.F.Barile and S. Razin. Academic Press, New York. pp. 63-102.

Bozeman, L.H., S.H.Kleven and R.B.Davis. (1984). Mycoplasma challenge studies in budgerigars (*Melopsittacus undulatus*) and chickens. Avian Dis., 28: 426-434.

Bradbury, J.M. (1972). Investigations into the production of *Mycoplasma gallisepticum* antigens. Ph.D. Thesis, University of Liverpool.

Bradbury, J.M. (1975). Concurrent infection of *M.synoviae* and *M.gallisepticum* in chickens. Proc.Conf.Taxon.Physiol.Anim.Mycoplasmas 3rd, Brno, pp. 106-115.

Bradbury, J.M. (1977). Rapid biochemical tests for characterization of the *Mycoplasmatales*. J. Clin. Microbiol., 5: 531-534.

Bradbury, J.M. (1982). The use of chicken antiserum for the identification of avian mycoplasmas by immunofluorescence. Avian Path., 11: 1113-121.

Bradbury, J.M. and F.T.W. Jordan. (1971a). The influence of pH of the culture medium on the sensitivity of *Mycoplasma gallisepticum* antigens for use in certain serological tests. J.Hyg.(Camb.), 69: 593-606.

Bradbury, J.M. and F.T.W. Jordan. (1971b). Investigation into rabbit infusion media for the growth of *Mycoplasma gallisepticum* antigens for inoculation into rabbits. J.Hyg.(Camb.), 69: 73-82.

Bradbury, J.M. and F.T.W.Jordan. (1972). Studies on the adsorption of certain medium proteins to *Mycoplasma gallisepticum* and their influence on agglutination and haemagglutination reactions. J.Hyg.(Camb.), 70: 267-278.

Bradbury, J.M. and J.McCarthy. (1981). Rupture of the digital flexor tendons of chickens after infection with *Mycoplasma iowae*. Vet.Rec., 109: 428-429.

Bradbury, J.M. and J.D.McCarthy. (1983). Pathogenicity of *Mycoplasma iowae* for chick embryos. Avian Path., 12: 483-496.

Bradbury, J. and M.Forrest. (1984). *Mycoplasma cloacale*, a new species isolated from a turkey. Int.J. Syst.Bact.,34: 389-392.

Bradbury, J.M. and J.D.McCarthy. (1984). *Mycoplasma iowae* infection in chicks. Avian Path., 13: 529-543.

Bradbury, J.M. and D.F.Kelly. (1991). *Mycoplasma iowae* infection in broiler-breeders. Avian Path., 20: 67-78.

Bradbury, J.M., M.Forrest and A.Williams. (1983). *Mycoplasma lipofaciens,* a new species of avian origin. Int. J.Syst.Bact., 33: 329-335.

Bradbury, J.M., F.T.W.Jordan, T.Shimizu, L.Stipkovits and Z.Varga. (1988a). *Mycoplasma anseris sp. nov.* found in geese. Int.J.Syst.Bact., 38: 74-76.

Bradbury, J.M., A.Ideris and T.T.Oo. (1988b). *Mycoplasma iowae* infection in young turkeys. Avian Path., 17: 149-171.

Bradley, L.D., D.B.Snyder and R.A.Van Deusen. (1988). Identification of species-specific and interspecies-specific polypeptides of *Mycoplasma gallisepticum* and *Mycoplasma synoviae.* Am.J.Vet.Res., 49: 511-515.

Bredt, W. (1973). Motility of mycoplasmas. Ann N.Y Acad Sci., 255: 246-250.

Bredt, W. (1983). Phase-contrast microscopy. In Methods in Mycoplasmology. Vol.I. Editors: S.Razin and J.G.Tully, Academic Press, New York. pp. 31-33.

Brunner, H., H.Krauss, H.Schaar and H.G.Schiefer. (1979). Electron microscopic studies on the attachment of *Mycoplasma pneumoniae* to guinea pig erythrocytes. Infect.Immun., 24: 906-911.

Buntz, B. (1987). Isolement et mise en evidence du pouvoir pathogene de *M.gallisepticum* chez l'oie (*Anser anser*). Thesis de doctorate. Universite de Bordeaux II.

Buntz, B., J.M.Bradbury, A.Vuillaume and D.Rousselot-Paillet. (1986). Isolation of *Mycoplasma gallisepticum* from geese. Avian Path., 15: 615-617.

Burger, H., Doss, M., Mannheim, W., and A. Schuler. (1967). Studies zur biochemischen Differenzierung von Mycoplasmen. Z. Med. Mikrobiol. Immunol., 153: 138-148.

Butler, M. (1969). Isolation and growth of mycoplasma in human embryo trachea culture. Nature, 224, 605-606.

Butler, M. and B.C.J.G.Knight. (1960). The survival of washed suspensions of mycoplasma. J.Gen.Microbiol., 22: 470-482.

Butler, M. and W.J.Ellaway. (1971). Growth and cytopathogenicity of mycoplasma in human and chicken trachea explants. J.Comp.Path., 81: 359-364.

Cassell, G.H., W.A.Clyde.Jr and J.K.Davis. (1985). Mycoplasmal respiratory infections. In: The Mycoplasmas, Vol IV. Editors: S.Razin and M.F.Barile. Academic Press, Inc, NewYork. pp. 65-106.

Chandler, D.K.F, S.Razin, E.B.Stephens, R.Harasawa and M.Barile. (1982). Genomic and phenotypic analyses of *Mycoplasma pneumoniae* strains. Infect.Immun., 38: 604-609.

Chanock, R.M., Hayflick, L. and M.F.Barile. (1962). Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natn. Acad. Sci., 48: 41-49.

Chaudhry, S. (1985). Preliminary investigations into the production of temperature-sensitive mutants of *Mycoplasma gallisepticum* by irradiation with ultraviolet light. M.Sc. Thesis. University of London.

Chen, Y.-Y. and D.C.Krause. (1988). Parasitism of hamster trachea epithelial cells by *Mycoplasma pneumoniae.* Infect. Immun., 56: 570-576.

Cherry, J.D. and D.Taylor-Robinson. (1970a). Large quantity production of chicken embryo tracheal organ culture and use in virus and mycoplasma studies. Appl.Microbiol., 19: 658-662.

Cherry, J.D. and D.Taylor-Robinson. (1970b). Growth and pathogenesis of *Mycoplasma mycoides var. capri* in chicken embryo tracheal organ cultures. Infect.Immun., 2, 431-438.

Cherry, J.D. and D.Taylor-Robinson. (1971). Growth and pathogenicity studies of *Mycoplasma gallisepticum* in chicken tracheal organ cultures. J.Med.Microbiol., 4: 441-449.

Cherry, J.D. and D.Taylor-Robinson. (1973). Mycoplasma pathogenicity studies in organ cultures. Ann.N.Y.Acad.Sci., 225: 290-303.

Clyde, W.A. (1964). Mycoplasma species identification based upon growth inhibition by specific antisera. J.Immun., 92: 958-965.

Clyde, W.A. (1983). Growth inhibition tests. In: Methods in Mycoplasmology. Vol.I. Editors: S.Razin and J.G.Tully, Academic Press, New York. pp. 405-410.

Clyde, W.A.Jr. and P.C.Hu. (1986). Antigenic determinants of the attachment protein of *Mycoplasma pneumoniae* shared by other pathogenic mycoplasma species. Infect.Immun., 51: 690-692.

Cole, R.M. (1983). Transmission electron microscopy: Basic techniques. In: Methods in Mycoplasmology, Vol.I. Editors: S.Razin and J.G.Tully, Academic Press, New York. pp. 43-50.

Cole, R.M. and T.J.Popkin. (1981). Electron microscopy and ultrastructure of bacteria. In: Manual of Methods for General Bacteriology, Chapter 4. Editors: P. Gerhardt, Am.Soc.Microbiol., Washington, D.C. pp.34-51.

Cole, R.M., J.G.Tully, T.J.Popkin and J.M.Bove. (1973). Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organisms (*Spiroplasma citri* gen.nov.,sp.nov.) cultured from "Stubborn" disease of citrus. J.Bact., 115: 367-386.

Collier, A.M. (1979). Mycoplasmas in organ culture. In: The Mycoplasmas, Vol.II. Editors: J.G.Tully and R.F.Whitcomb, Academic Press, New York. pp. 475-493.

Collier, A.M. and W.A.Clyde.Jr. (1971). Relationships between *Mycoplasma pneumoniae* and human respiratory epithelium. Infect.Immun., 3: 694-701.

Collier, A.M. and J.B. Baseman. (1973). Organ culture techniques with mycoplasmas. Ann.N.Y.Acad.Sci., 225: 277-289.

Collier, A.M. and J.L.Carson. (1983). Tracheal organ cultures as modelsin pathogenicity studies. In: Methods in Mycoplasmology, Vol.II. Editors: S.Razin and J.G. Tully, Academic Press, New York. pp. 331-335.

Collier, A.M., W.A.Clyde Jr. and F.W.Denny. (1969). Biologic effects of *Mycoplasma pneumoniae* and other mycoplasmas from man on hamster tracheal organ culture. Proc.Soc.Exp.Biol.Med., 132: 1153-1158.

Collier, A.M., W.A.Clyde Jr. and F.W. Denny. (1971). *Mycoplasma pneumoniae* in hamster tracheal organ culture: Immunofluorescent and electron microscopic studies. Proc.Soc.Exp.Biol.Med., 136: 569-573.

Coons, A.H., H.J.Creech and R.N.Jones. (1941). Immunological properties of an antibody containing a fluorescent group. Proc.Soc.Exp. Biol.Med., 47: 200-202.

Davidson, M.K., S.E.Ross, J.R.Lindsey and G.H.Cassell. (1988). Colony opacity, hemadsorption, hemolysis and mitogenicity are not associated with virulence of *Mycoplasma pulmonis.* Infect. Immun., 56: 2169-2173.

Del Giudice, R.A., N.F.Robillard and T.R.Careki. (1967). Immunofluorescence identification of mycoplasma on agar by use of incident illumination. J.Bact., 93: 1205-1209.

Del Giudice, R.A., J.G.Tully, D.L.Rose and R.M.Cole. (1985). *Mycoplasma pirum sp.nov.*, a terminal structured *Mollicute* from cell cultures. Int.J.Syst.Bact., 35, 285-291.

Dienes, L. (1963). Comparative morphology of L-forms and pleuropneumonia-like organisms. In: 8th Internat. Congress for Microbiol, Montreal, 1962. Recent Prog. Microbiol., 8: 511-517.

Dienes, L. and H.J.Weinberger. (1951). The L-form of bacteria. Bact. Reviews, 15: 245-288.

Dierks, R.E., J.A.Newman and B.S.Pomeroy. (1967). Characterization of avian mycoplasma. Ann.N.Y.Acad.Sci., 143: 170-189.

Dodd, S. (1905). Epizootic pneumo-enteritis of the turkey. J.Comp.Path.Ther., 18: 239-245. Cited by Jordan, (1979).

Dupiellet, J-P. (1984). *Mycoplasma et acholeplasmes* des palmipedes a foie gras: isolement, caracterisation, etude du role dans la pathologie. Rapport de D.E.A. de Pathologie, Universite de Bordeaux II.

Dupiellet, J-P. (1988). Mycoplasmes de l'oie et du canard: contribution a l'etude serologique et moleculaire de souches apparentees a *Mycoplasma gallisepticum*. These de Doctorate. Universite de Bordeaux II.

Dupiellet, J-P., A.Vuillaume, D.Rousselot, J.M.Bove and J.M.Bradbury. (1990). Serological and molecular studies on *Mycoplasma gallisepticum* strains. Proceed. of the 7th International Congress of the IOM, Baden, 1988. In: Zbl. Bakt. Suppl. 20. Gustav Fischer Verlag. Stuttgart. New York. pp. 859-864.

Dykstra, M.J., S.Levisohn, O.J.Fletcher and S.H.Kleven. (1985). Evaluation of cytopathologic changes induced in chicken tracheal epithelium by *Mycoplasma gallisepticum in vivo* and *in vitro*. Am.J.Vet.Res., 46: 116-122.

Edward, D.G.ff. (1960). Biology of the pleuropneumonia-like organisms. Ann.N.Y.Acad.Sci., 79: 309-311.

Edward, D.G.ff. (1971). Determination of sterol requirement for *Mycoplasmatales*. J.Gen. Microbiol., 69: 205-210.

Edward, D. G. ff. and W.A.Fitzgerald. (1951). Cholesterol in the growth of the pleuropneumonia group. J.Gen.Microbiol., 5: 576-586.

Edward, D. G. ff. and W.A.Fitzgerald. (1954). Inhibition of growth of pleuropneumonia-like organisms by antibody. J. Path.Bact., 68: 23-30.

Edward, D.G.ff. and E.A. Freundt. (1967). Proposal for *Mollicutes* as name of the class established for the order *Mycoplasmatales*. Int. J.Sys.Bact., 17: 267-268.

Edward, D.G.ff. and W.B.Moore. (1975). A method for determining utilization of glucose by mycoplasmas. J.Med.Microbiol., 8: 451-454.

Engelhardt, J.A. and M.G.Gabridge. (1977). Effect of squamous metaplasia on infection of hamster trachea organ cultures with *Mycoplasma pneumoniae*. Infect.Immun., 15: 647-655.

Erickson, B.Z., R.F.Rose, D.L.Rose, J.G.Tully ad J.M.Bove. (1986). *Mycoplasma hyopharyngis*, a new species from swine. Int.J.Sys.Bact., 36: 55-59.

Erno, H. (1977). Mycoplasmas: use of polyvalent antisera for identification by indirect immunofluorescence. Acta Vet.Scand., 18: 176-188.

Erno, H. and K.Jurmanova. (1973). Bovine Mycoplasmas: serological studies by double immunodiffusion, growth precipitation and growth inhibition. Acta Vet. Scand., 14: 524-537.

Erno, H. and L.Stipkovits. (1973). Bovine mycoplasmas: cultural and biochemical studies. II. Acta Vet. Scand., 14: 450- 463.

Fabricant, J. and E.A.Freundt. (1967). Importance of extension and standardization of laboratory tests for the identification and classification of mycoplasma. Ann.N.Y.Acad.Sci., 143: 50-58.

Fawzia, M.M. (1976). Studies on the isolation and characterization of mycoplasma and acholeplasma in ducks. M.V.Sci.Thesis, Cairo University.

Feldner, J.and W.Bredt. (1983). Analysis of polypeptides of mutants of *Mycoplasma pneumoniae* that lack the ability to haemadsorb. J.Gen.Microbiol., 129: 841-848.

Feldner, J., U.Gobel and W.Bredt. (1982). *Mycoplasma pneumoniae* adhesion localized to tip structure by monoclonal antibody. Nature, 298: 765-767.

Forrest, M. (1982). Characterization of three new species of avian mycoplasma. Ph.D. Thesis, University of Liverpool.

Forrest, M. and J.Bradbury. (1984). *Mycoplasma glycophilum*, a new species of avian origin. J.Gen.Microbiol., 130: 597-603.

Forsyth, M., M.Tourtellotte and S.Geary. (1990). Characterization of a hemagglutinin from *Mycoplasma gallisepticum.* IOM Letters, Program and abstracts of the 8th International Congress of the IOM,Istanbul-Turkey, Vol. 1: Abstract No. P161.

Fox, G.E., E.Stackebrandt, R.B.Hespell, J.Gibson, J.Maniloff, T.A.Dyer, R.S.Wolfe, W.E.Balch, R.S.Tanner, L.J.Magrum, L.B.Zablen, R.Blakemore, R.Gupta, L.Bonen, B.J.Lewis, D.A.Stah K.R.Luehrsen, K.N.Chen and C.R.Woese. (1980). The phylogeny of prokaryotes. Science, 209: 457-463.

Freundt, E.A. (1983a). Culture media for classic mycoplasmas. In: Methods in Mycoplasmology, Vol. I. Editors: S.Razin and J.G.Tully, Academic Press, NewYork. pp. 127-135.

Freundt, E.A. (1983b). Principles of mycoplasma classification and taxonomy. In: Methods in Mycoplasmology, Vol.I. Editors: S. Razin and J.G.Tully, Academic Press, New York. pp. 9-13.

Freundt, E.A. (1983c). Proteolytic activity. In: Methods in Mycoplasmology, Vol.I. Editors: S. Razin and J.G.Tully, Academic Press, New York. pp. 367-371.

Freundt, E.A. and D.G.ff.Edward. (1979). Classification and taxonomy. In: The Mycoplasmas, Vol. I. Editors: M.F.Barile and S.Razin. Academic Press, New York. pp. 1-41.

Freundt, E.A., H.Erno, F.T.Black, A.Krogsgaard-Jensen and S.Rosendal. (1973). Evaluation of reference reagents for mycoplasmas. Ann.N.Y.Acad.Sci., 225: 161-171.

Freundt, E A., Erno, H., and R.M.Lemcke. (1979). Identification of mycoplasmas. In: Methods of Microbiology, Vol.13. Editors: T.Bergan and J.R.Norris. Academic Press. New York. pp. 377-434.

Freundt, E.A., R.F.Whitcomb, M.F.Barile, S.Razin and J.G.Tully. (1984). Proposal for elevation of family *Acholeplasmataceae* to ordinal rank: *Acholeplasmatales* . Int.J.Syst.Bact., 34: 346-349.

Frey, M.L., S.T.Hawk and P.A.Hale. (1972). A division by micro-complement fixation tests of previously reported avian mycoplasma serotypes into identification groups. Avian Dis., 16: 780-792.

Frlis, N.F. (1971). Mycoplasmas cultivated from the respiratory tract of Danish pigs. Acta Vet. Scand., 12: 69-79.

Gabridge, M.G. (1979). Hamster tracheal organ cultures as model for infection and toxicology studies. Proc. Exp. Tumor Res., 24: 85-95.

Gabridge, M.G. (1984). Respiratory tract organ cultures to assay attachment and pathogenicity of mycoplasmas. Ann.Microbiol.(Inst.Pasteur), 135A: 33-38.

Gabridge, M.G. and Y.D. Barden Stahl. (1978). Role of cell-associated pathogen metabolism in infection of tracheal explants by *Mycoplasma pneumoniae.* Med.Microbiol.Immun., 165: 153-161.

Gabridge, M.G., D.Taylor-Robinson, H.A.Davis and R.R.Dourmashkin. (1979). Interaction of *Mycoplasma pneumoniae* with human lung fibroblasts: characterization of the *in vitro* model. Infect.Immun., 25: 446-454.

Gadeau, A.P., C. Mouches and J.M.Bove. (1986). Probable insensitivity of mollicutes to rifampin and characterization of spiroplasmal DNA-dependent RNA polymerase. J.Bact., 166: 824-828.

Gardella, R.S. and R.A.DelGiudice. (1983). Hemagglutination, hemadsorption and hemolysis. In: Methods in Mycoplasmology, Vol. I. Editors: S.Razin and J.G.Tully, Academic Press, New York. pp.379-384.

Gibbons, N.E. and R.G.E. Murray. (1978). Proposal concerning the higher taxa of bacteria. Int.J.Syst.Bact., 28: 1-6.

Giebel, J., J. Meier, A.Binder, J.Flosedorf, J.B.Poveda, R.Schmidt and H.Kirchhoff. (1991). *Mycoplasma phocarhinis sp. nov.* and *Mycoplasma phocacerebrale sp.nov.*, two new species from harbor seals (*Phoca vitulina* L.). Int.J.Sys.Bact., 41: 39-44.

Gourlay, R.N., S.G.Wyld and R.H.Leach. (1977). *Mycoplasma alvi*, a new species from bovine intestinal and urogenital tracts. Int.J.Syst.Bacteriol., 27: 86-96.

Gourlay, R.N., S.G.Wyld and R.H.Leach. (1978). *Mycoplasma sualvi*, a new species from the intestinal and urogenital tracts of pigs. Int.J.Syst.Bact., 28: 289-292.

Hansen, E.J., R.M.Wilson and J.B.Baseman. (1979). Isolation of mutants of *Mycoplasma pneumoniae* defective in hemadsorption. Infect.Immun.,23: 903-906.

Hansen, E.J., R.M.Wilson, W.A.Clyde.Jr.and J.B.Baseman. (1981). Characterization of hemadsorption-negative mutants of *Mycoplasma pneumoniae.* Infect.Immun., 32: 127-136.

Hayat, M.A. (1972). In: Basic electron microscopy techniques. Fixation by immersion. Editor: Van Nostrand Reinhold Company, New York, Cincinnati, Toronto, London, Melbourne. pp. 1-26.

Hill, A.C. (1991). *Mycoplasma oxoniensis*, a new species isolated from Chinese hamster conjunctivas. Int.J.Syst.Bact., 41: 21-25.

Hirano, H.,E.Hayatsu, K.Kume, Y.Kawakubo and S.Nakagawa. (1978). Pathogenicity of *Mycoplasma synoviae* in chicken tracheal organ cultures. Jap.J.Vet.Sci., 40: 147-156.

Howard, C.J. and L.H.Thomas. (1974). Inhibition by *Mycoplasma dispar* of ciliary activity in tracheal organ cultures. Infect.Immun., 10: 405-408.

Hu,P.C., Collier, A.M and J.B.Baseman. (1977). Surface parasitism by *Mycoplasma pneumoniae* of respiratory epithelium. J.Exp.Med., 145: 1328-1343.

Hu, P.C., R.M.Cole, Y.S.Huang, A.M.Collier and W.A.Clyde.Jr. (1982). *Mycoplasma pneumoniae*: role of a surface protein in the attachment organelle. Science, 216: 313-315.

Hu, P.C., W.A.Jr.Clyde, and A.M.Collier. (1984). Conservation of pathogenic mycoplasma antigens. Isr.J.Med.Sci., 20: 916-919.

Hu, P.C., A.M.Collier and W.Jr.Clyde. (1990). Metabolic alteration in tracheal organ cultures following *Mycoplasma pneumoniae* infection. IOM letter, Program and Abstracts of the 8th International Congress of the IOM, Istanbul-Turkey, Vol.1: Abstract No. W13/5.

Huggett, A.St.G. and D.A.Nixon. (1957). Use of glucose oxidase, peroxidase, and O-dianisidine in determination of blood and urinary glucose. Lancet, 2: 368-370.

International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*. (1988). Minutes of the meeting, 25 and 28 August, 1986. Int.J.Syst.Bact.,38: 226-230.

International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*. (1991a). Minutes of the meeting, 1 and 2 June, 1988. Int.J.Syst.Bact., 41: 180-182.

International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*. (1991b). Minutes of the meeting, 7 and 8 July, 1990. Int.J.Syst.Bact., 41: 333-336.

Johnson, G.D. and G.M.de.C.N.Araujo. (1981). A simple method of reducing the fading of immunofluorescence during microscopy. J.Immun.Methods, 43: 349-350.

Johnson, J.L. (1984). Nucleic acids in bacterial classification. In: Bergey's Manual of Systematic Bacteriology, Vol.1. Editors: N.R.Kreig, Williams and Wilkins, Baltimore and London. pp. 8-11

Jordan, F.T.W. (1973). A combined growth-inhibition and growth-precipitation test for the typing of avian mycoplasma. Res.Vet.Sci., 14: 387-389.

Jordan, F.T.W. (1979). Avian mycoplasmas. In: The Mycoplasmas, Vol.II. Editors: J.G.Tully and R.F.Whitcomb. Academic Press, NewYork. pp. 1-48.

Jordan, F.T.W. (1983). Recovery and identification of avian mycoplasmas. In: Methods in Mycoplasmology, Vol. II. Editors: S.Razin and J.G.Tully, Academic Press, New York. pp. 69-79.

Jordan, F.T.W. (1985). Gordon Memoral Lecture: People, poultry and pathogenic mycoplasmas. Brit. Poult.Sci., 26, 1-15.

Jordan, F.T.W. (1990). Avian Mycoplasmoses. In: Poultry Diseases, 3rd Edition. Editor: F.T.W.Jordan, Bailliere Tindall, London. pp.74-85.

Jordan, F.T.W. and P.Kulasegaram. (1968). Serological tests for the detection of antibodies to *Mycoplasma gallisepticum* in chicken and turkeys. J.Hyg.(Camb.), 66: 249-267.

Jordan, F.T.W. and M.M. Amin. (1980). A survey of mycoplasma infections in domestic poultry. Res.Vet.Sci., 28: 96-100.

Jordan, F.T.W., H.Erno, G.S.Cottew, K.H.Hinz and L.Stipkovits. (1982). Characterization and taxonomic description of five mycoplasma serovars (serotypes)

of avian origin and their elevation to species rank and further evaluation of the taxonomic status of *Mycoplasma synoviae*. Int.J.Syst.Bact., 32: 108-115.

Kahane, I., S.Tucker and J.B.Baseman. (1985). Detection of *Mycoplasma pneumoniae* adhesin (P1) in the nonhemadsorbing population of virulent *Mycoplasma pneumoniae*. Infect. Immun., 49: 457-458.

Khan, M.I. and R.Yamamoto. (1989). Differentiation of the vaccine F-strain from other strains of *Mycoplasma gallisepticum* by restriction endonuclease analysis. Vet.Microb., 19: 167-174.

Kirchhoff, H.,P.Beyene, M.Fischer, J.Flobdorf, J.Heitmann, B.Kbattab, D.Lopatta, R.Rosengarten, G.Seidel and Cb.Yousef. (1987). *Mycoplasma mobile sp.nov.*, a new species from fish. Int.J.Syst.Bacteriol., 37: 192-197.

Kleckner, A.L. (1960). Serotypes of avian pleuropneumonia-like organisms. Am.J.Vet.Res., 21: 274-280.

Kleven, S.H., C.S.Eidson and O.J.Fletcher. (1978). Airsacculitis induced in broilers with a combination of *Mycoplasma gallinarum* and respiratory viruses. Avian Dis., 22: 707-716.

Kleven, S.H., G.F.Browing, D.M.Bulach, E.Ghiocas, C.J.Morrow and K.G. Whithear. (1988a). Examination of *Mycoplasma gallisepticum* strains using restriction endonuclease DNA analysis and DNA-DNA hybridisation. Avian Path., 17: 559-570

Kleven, S.H.,C.J.Morrow and K.G.Whithear. (1988b). Comparison of *Mycoplasma gallisepticum* strains by hemagglutination-inhibition and restriction endonuclease analysis. Avian Dis., 32: 731-741.

Klieneberger-Nobel, E. (1960). Classification of PPLO. Ann.N.Y.Acad.Sci., 79: 483-484.

Koshimizu, K. and T.Magaribuchi. (1977). Isolation of ureaplasmas (T-mycoplasma) from the chicken and jungle-fowl. Japn.J.Vet.Sci., 39: 195-199.

Koshimizu, K. R.Harasawa, I.-J., Pan, H.Kotani, M.Ogata, E.B.Stephens and M.F.Barile. (1987). *Ureaplasma gallorale sp. nov.* from oropharynx of chickens. Int.J.Syst.Bact., 37: 333-338.

Krause, D.C.and J.B.Baseman. (1982). *Mycoplasma pneumoniae* proteins that selectively bind to host cells. Infect.Immun., 37: 382-386.

Krause, D.C., D.K. Leith, R.M.Wilson and J.B.Baseman. (1982). Identification of *Mycoplasma pneumoniae* proteins associated with hemadsorption and virulence. Infect.Immun., 35: 809-817.

Lam, K.M., J.Rosen and H.E.Adler. (1984). Temperature-sensitive mutants of *Mycoplasma gallisepticum.* J.Comp.Path., 94: 1-8.

Leith, D.K., E.J.Hansen, R.M.Wilson, D.C.Krause and J.B.Baseman. (1983). Hemadsorption and virulence are separable properties of *Mycoplasma pneumoniae* . Infect.Immun., 39: 844-850.

Lemcke, R.M. (1972). Osmolar concentration and fixation of mycoplasmas. J.Bact., 110: 1154-1162.

Levisohn, S., Y.Yegana, I.Hod and A.Herz. (1983). A correlative *in vivo* study of the surface morphology and colonisation of the chicken trachea infected by *Mycoplasma gallisepticum* strains R and F. Avian Path., 12: 247-261.

Levisohn, S., J.R.Glisson and S.H.Kleven. (1985). *In ovo* pathogenicity of *Mycoplasma gallisepticum* strains in the presence and absence of maternal antibody. Avian Dis., 29: 188-197.

Levisohn, S., M.J.Dykstra, M.Y.Lin and S.H. Kleven. (1986). Comparison of *in vivo* and *in vitro* methods for pathogenicity evaluation for *Mycoplasma gallisepticum* in respiratory infection. Avian Dis., 15: 233-246.

Lin, M.Y. and S.H.Kleven. (1982). Pathogenicity of two strains of *Mycoplasma gallisepticum* in turkeys. Avian Dis., 26: 360-364.

Lipman, R.P. and W.A.Clyde Jr. (1969). The interrelationship of virulence, cytadsorption, and peroxide formation in *Mycoplasma pneumoniae* (34061). Proc.Soc.Exp.Biol.Med., 131: 1163-1167.

Liu, C. (1957). Studies on primary atypical pneumonia. I. Localization, isolation and cultivation of a virus in chick embryos. J. Exp.Med., 106: 455-466.

Livingston, C.W. (1972). Isolation of T-strain of mycoplasma from Texas feedlot cattle. Am.J.Vet.Res., 33: 1925-1929.

MacOwan, K.J., C.J.Randall and T.F.Brand. (1983). Cloacal infection with *Mycoplasma gallisepticum* and the effect of inoculation with H120 infectious bronchitis vaccine virus. Avian Path., 12: 497-503.

Madden, D.L., W.H.Henderson and A.E. Moses. (1967). Case report: Isolation of *Mycoplasma gallisepticum* from Bobwhite quail (*Colinus virginianus*). Avian Dis., 11: 378-380.

Manchee, R.J. and D.Taylor-Robinson. (1968). Haemadsorption and haemagglutination by mycoplasmas. J.Gen.Microbiol., 50: 465-478.

Maniloff, J. and H.J.Morowitz. (1967). Ultrastructure and life cycle of *Mycoplasma gallisepticum* A5969. Ann.N.Y.Acad.Sci., 143: 59-65.

Maniloff, J. and H.Morowitz. (1972). Cell biology of the mycoplasmas. Bact.Rev., 36: 263-290.

Maniloff, J., H.J.Morowitz and R.J.Barnett. (1965). Ultrastructure and ribosomes of *Mycoplasma gallisepticum*. J.Bact.,90: 193-204.

Markham, F.S. and S.C.Wong. (1952). Pleuropneumonia-like organisms in the etiology of turkey sinusitis and chronic disease of chickens. Poult.Sci., 31: 902-904.

Matsuyama, T. (1974). Point inoculation of cultivated tracheal mucous membrane with bacteria. J.Infect.Dis., 130: 508-514.

McClenaghan, M. (1982). Studies on avian mycoplasma mixtures and an investigation into the pathogenicity of serotype I. Ph.D. Thesis, University of Liverpool.

McClenaghan, M., J.M.Bradbury and J.N.Howse. (1981). Embryo mortality associated with avian mycoplasma serotype I. Vet.Rec., 108: 459-460.

McDowell, E.M. and B.F.Trump. (1976). Histologic fixatives suitable for diagnostic light and electron microscopy. Arch.Pathol.Lab.Med., 100: 405-414.

Mew, A.J., G.Ionas, J.K.Clarke, A.J.Robinson and R.B.Marshall. (1985). Comparison of *Mycoplasma ovipneumoniae* isolates using bacterial restriction endonuclease DNA analysis and SDS-PAGE. Vet. Microb., 10: 541-548.

Miles, A.A., S.S. Misra and J.O.Irwin. (1938). The estimation of bactericidal power of the blood. J.Hyg. (Camb.), 38: 732-749.

Miller, J.H. (1972). Experiments in molecular genetics. Cold Spring Harbour Publication, pp.121-122.

Morrow, C.J., K.G.Whithear and S.H.Kleven. (1990). Restriction endonuclease analysis of *Mycoplasma synoviae* strains. Avian Dis., 34: 611-616.

Murakami, T. and T.Matsuyama. (1980). Growth of *Bacteriodes fragilis* inoculated on rabbit tracheal explant in an atmospheric environment. J.Infect. Dis., 142: 332-337.

Muse, K.E., D.A.Powell and A.M. Collier. (1976). *Mycoplasma pneumoniae* in hamster tracheal organ culture studied by scanning electron microscopy. Infect.Immun., 13: 229-237.

Neimark, H. (1979). Phylogenetic relationships between mycoplasmas and other prokaryotes. In: The Mycoplasmas, Vol. I. Editors: M.F.Barile and S.Razin. Academic Press, New York. pp. 43-61.

Neimark, H.C. and C.S.Lange. (1990). Pulse-field electrophoresis indicates full-length mycoplasma chromosomes range widely in size. Nucleic Acids Res., 18: 5443-5448.

Nelson, J.B. (1936). Studies on an uncomplicated coryza of the domestic fowl. V. A coryza of slow onset. J.Exp.Med., 63: 509- 513.

Nelson, J.B. (1939). Growth of the fowl coryza bodies in tissue culture and blood agar. J.Exp.Med., 69: 199-209.

Nicol, C.S. and D.G.ff. Edward. (1953). Role of organisms of the pleuropneumonia group in human genital infections. Br.J.Vener.Dis., 29: 141-150.

Nonomura, I and S.Sato. (1975). Interference of *Mycoplasma gallisepticum* with multiplication of Newcastle disease virus in chickens. Avian Dis.,19: 603-607.

Nonomura, I. and Y.Imada. (1982). Temperature-sensitive mutants of *Mycoplasma synoviae.* I. Production and selection of a nonpathogenic but immunogenic clone. Avian Dis., 26: 763-775.

Oriel, C.A. (1976). *Mycoplasma synoviae* in tracheal organ cultures. Report on project for degree of BSc., Liverpool Polytechnic.

Osborn, O.H. and B.S. Pomeroy. (1958). Isolation of the agent of infectious sinusitis of turkeys from naturally infected pheasants. Avian Dis., 2: 370-372.

Pellegrin, J-L., J.Maugein, M-T.Clerc, B. Leng, J-M. Bove and C.Bebear. (1990). Activity of Rifampin against *Mollicutes*, *Clostridia* and *L* Forms. In: Proceed. of the 7th International Congress of the IOM, Baden, Zbl.Bakt. Suppl.20. Gustav. Fischer Verlag. Stuttgart. New York. pp.800.

Power, J. and F.T.W.Jordan. (1973). The virulence of *Mycoplasma gallisepticum* for embryonated fowl eggs. Res.Vet.Sci., 14: 259-261.

Power, J. and F.T.W.Jordan. (1976). A comparison of the virulence of three strains of *Mycoplasma gallisepticum* and one strain of *Mycoplasma gallinarum* in chicks, turkey poults, tracheal organ cultures and embryonated fowl eggs. Res.Vet.Sci., 21: 41-46.

Pyle, L.E., L.N.Corcoran, G.C.Benjamin, A.D.Bergemann, J.C.Whitley and L.R.Finch. (1988). Pulsed-field electrophoresis indicates larger-than-expected sizes for mycoplasma genomes. Nucleic Acids Res., 16: 6015-6025.

Radestock, U. and W.Bredt. (1977). Motility of *Mycoplasma pneumoniae.* J.Bact., 129: 1435.

Razin, S. (1969). Structure and function in mycoplasma. Ann.Rev. Microbiol., 23: 317-356.

Razin, S. (1978). The Mycoplasmas. Microbiol. Reviews, 42: 414-470.

Razin, S. (1983a). Identification of mycoplasma colonies. In Methods in Mycoplasmology, Vol. I. Editors: S.Razin and J.G.Tully, Academic Press, NewYork. pp. 83-88.

Razin, S. (1983b). Morphology and Ultrastructure: Introductory comments. In: Methods in Mycoplasmology, Vol.I. pp. 29-30.

Razin, S. (1985). Mycoplasma adherence. In: The Mycoplasmas, Mycoplasma pathogenicity, Vol.IV. Editors: S.Razin and M.F.Barile, Academic Press, Inc. NewYork. pp. 161-202.

Razin, S. (1986). Mycoplasmal adhesins and lectins. In: Microbial lectins and agglutinins: properties and biological activity. Editor: D.Mirelman. pp. 217-235

Razin, S. and J.G.Tully. (1970). Cholesterol requirement of *Mycoplasmas*. J.Bact., 102: 306-310.

Razin, S., I.Kahane, M.Banai and W.Bredt. (1981). Adhesion of mycoplasmas to eukaryotic cells. Ciba Found.Symp., 80: 98-118.

Razin, S., R.Harasawa and M.F.Barile. (1983a). Cleavage patterns of the mycoplasma chromosome, obtained by using restriction endonucleases, as indicators of genetic relatedness among strains. Int.J.Syst.Bacteriol., 33: 201-206.

Razin, S., J.G.Tully, D.L.Rose and M.F.Barile. (1983b). DNA cleavage patterns as indicators of genotypic heterogeneity among strains of *Acholeplasma* and *Mycoplasma* species. J.Gen.Microb., 129: 1935-1944.

Reece, R.C., I.Ireland and D.A.Barr. (1986). Infectious sinusitis associated with *Mycoplasma gallisepticum* in game birds. Aust.Vet.J., 63: 167.

Reed, S.E. (1971). The interaction of mycoplasma and influenza viruses in tracheal organ cultures. J.Infect. Dis., 124: 18-25.

Reed, S.E. (1972). Viral enhancement of mycoplasma growth in tracheal organ cultures. J.Comp.Path., 82: 267-278.

Rhoades, K.R. (1977). Turkey sinusitis: synergism between *Mycoplasma synoviae* and *Mycoplasma meleagridis*. Avian Dis., 21: 670-674.

Rhoades, K.R. (1981a). Pathogenicity of strains of the IJKNQR group of avian mycoplasmas for turkey embryos and poults. Avian Dis., 25: 104-111.

Rhoades, K.R. (1981b). Turkey airsacculitis: effects of mixed mycoplasma infections. Avian Dis., 25: 131-135.

Rhoades, K.R. (1985) Selection and comparison of *Mycoplasma synoviae* hemagglutinating and nonhemagglutinating variants. Avian Dis., 29: 1170-1176.

Roberts, D.H. (1971). Interaction of porcine mycoplasmas with fresh animal serum. J. Hyg.(Camb.), 69: 361-368.

Roberts, D.H., O.M.Olesiuk and H.V.Roekel. (1967). Immunologic response of fowl to *Mycoplasma gallisepticum* and its relationship to latent infection. Am.J.Vet.Res., 28: 1135-1152.

Robinson, I.M. and E.A.Freundt. (1987). Proposal for an amended classification of anaerobic *Mollicutes.* Int.J.Syst.Bact., 37: 78-81.

Robertson, J., M.Gomersall and P.Gill. (1975a). *Mycoplasm hominis:* growth, reproduction, and isolation of small viable cells. J.Bact., 124: 1007-1018.

Robertson, J., M.Gomersall and P.Gill. (1975b). Effect of preparatory techniques on the gross morphology of *Mycoplasma hominis.* J.Bact., 124: 1019-1022.

Rose, D.L., J.P.Kocka, N.L.Somerson, J.G.Tully, R.F.Whitcomb, P.Carle, J.M.Bove, D.E.Colflesh and D.L.Williamson. (1990). *Mycoplasma lactucae sp.nov.*, a sterol-requiring mollicute from a plant surface. Int.J.Sys. Bact., 40: 138-142.

Rosendal, S. and F.T.Black. (1972). Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. Acta. Path.Microbiol.Scand., Sec.B., 80: 615-622.

Rottem, S. (1983). Harvest and washing of mycoplasmas. In: Methods in Mycoplasmology, Vol.I. Editors: S. Razin and J.G.Tully, Academic Press, New York. pp. 221-223.

Rottem, S., C.Linker, and T.H.Wilson. (1981). Proton motive force across the membrane of *Mycoplasma gallisepticum* and its possible role in cell volume regulation. J.Bact., 145: 1299-1304.

Sabry, M.Z., A.M.Soliman, M.M.Fawzia and A.A.Ahmed. (1990). Isolation, identification and pathogenicity of mycoplasmas from ducks in Egypt. IOM Letters, Program and Abstracts of the 8th International Congress of the IOM, Istanbul-Turkey, Vol. 1: Abstract No. P170.

Salih, B.A. and R.F.Rosenbush. (1988). Attachment of *Mycoplasma bovoculi* to bovine conjunctival epithelium and lung fibroblasts. Am.J.Vet.Res., 49: 1661-1664.

Sambrook, J.,E.F.Fritsch and T.Maniatis. (1989). Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, 2nd Edit., NY, Vol. 3, Appendix B, p.23.

Santha, M., K.Lukacs, K.Burg, S.Bernath, I.Rasko and L.Stipkovits. (1988). Intraspecies genotypic heterogeneity among *Mycoplasma gallisepticum* strains. Appl.Enviroment.Microb., 54: 607-609.

Schimke, R.T. and M.F.Barile. (1963). Arginine metabolism in pleuropneumonia-like organisms isolated from mammalian cell culture. J.Bact., 86: 195-206.

Shimizu, T. and H.Nagatomo. (1989). An adhesion-hemadsorption test for screening and identification of *Mycoplasma gallisepticum.* Jpn.J.Vet.Sci., 51: 206-208.

Shimizu, T., H.Erno and H.Nagatomo. (1978). Isolation and characterization of *Mycoplasma columbinum* and *Mycoplasma columborale*, two new species from pigeons. Int.J.Syst.Bact., 28: 538-546.

Shepard, M.C. and D.R.Howard. (1970). Identification of "T" mycoplasmas in primary agar cultures by means of a direct test for urease. Ann.N.Y.Acad. Sci., 174: 809-819.

Shepard, M.C., C.D.Lunceford, D.K.Ford, R.H.Purcell, D.Taylor-Robinson, S.Razin and F.T.Black. (1974). *Ureaplasma urealyticum gen.nov.*,

sp.nov.: proposed nomenclature for the human T (T strain) mycoplasmas. Int.J.Syst.Bact., 24: 160-171.

Sobeslavsky, O, B.Prescott and R.M.Chanock. (1968). Adsorption of *Mycoplasma pneumoniae* to neuraminic acid receptors of various cells and possible role in virulence. J.Bact., 96: 695-705.

Spurr, A.R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. J.Ultrastruct.Res., 26: 31-43.

Stadtlander, C. and H.Kirchhoff. (1988). The effect of *Mycoplasma mobile* 163 K on the ciliary epithelium of tracheal organ cultures. Zbl.Bakt.Hyg., A269: 355-365.

Stadtlander, C. and H.Kirchhoff. (1990). Surface parasitism of the fish mycoplasma *Mycoplasma mobile* 163 K on tracheal epithelial cells. Vet.Microbiology, 21: 339-343.

Stanbridge, E.J. and M. E.Reff. (1979). The molecular biology of mycoplasmas. In: The Mycoplasmas, Vol.I. Editors: M.F.Barile and S.Razin. Academic Press, NewYork. pp. 157-185.

Steinberg, P., Horswood, R.L.and R.M.Chanock. (1969). Temperature-sensitive mutants of *Mycoplasma pneumoniae*. I. *In vitro* biologic properties. J.Infect.Dis., 120: 217-224.

Stipkovits, L. and A.Rashwan. (1976). Isolation of ureaplasmas from chickens. Proc. Soc.Gen.Microbiol. 3: 158.

Stipkovits, L., El-Ebeedy, Kisary, J. and L.Varga. (1975). Mycoplasma infection of geese I. Incidence of mycoplasma and acholeplasmas in geese. Avian Path., 4: 35-43.

Stipkovits, L., A.Rashwan, J.Takacs and K.Lapis. (1978). Occurrence of ureaplasmas in swine semen. Zbl.Vet. Med.Reihe B, 25: 605-608.

Subcommittee on the Taxonomy of *Mollicutes*. (1979). Proposal of minimal standards for descriptions of new species of the class *Mollicutes*. Int.J.Syst.Bacteriol., 29: 172-180.

Su, C.J., V.V.Tryon and J.B.Baseman. (1987). Cloning and sequence analysis of cytadhesin P1 gene from *Mycoplasma pneumoniae*. Infect.Immun., 55: 3023-3029.

Tajima, M., and T.Yagihashi. (1982). Interaction of *Mycoplasma hyopneumoniae* with the porcine respiratory epithelium as observed by electron microscopy. Infect.Immun., 37: 1162-1169.

Tajima, M., T.Nunoya and T.Yagihashi. (1979). An ultrastructural study on the interaction of *Mycoplasma gallisepticum* with the chicken tracheal epithelium. Am.J.Vet.Res., 40: 1009-1014.

Tajima, M., T.Yagihashi and Y.Miki. (1982). Capsular material of *Mycoplasma gallisepticum* and its possible relevance to the pathogenic process. Infect.Immun., 36: 830-833.

Takagi, H. and A.Arakawa. (1980). The growth and cilia-stopping effect of *Mycoplasma gallisepticum* 1RF in chicken tracheal organ cultures. Res.Vet. Sci., 28: 80-86.

Taylor-Robinson, D. and J.D.Cherry. (1972). A non-pathogenic mycoplasma inhibiting the effect of a pathogenic mycoplasma in organ culture. J.Med.Microbiol., 5: 291-298.

Taylor-Robinson, D., R.H.Purcell, D.C.Wong and R.M. Chanock. (1966). A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. J.Hyg.Camb., 64: 91-104.

Tiong, S.K. (1978). Isolation of *Mycoplasma gallisepticum* from sinuses of three quail (*Coturnix coturnix japonica*). Vet Rec., 103: 539.

Tiong, S.K. (1990). *Mycoplasmas* and *acholeplasmas* isolated from ducks and their possible association with pasteurellas. Vet.Rec., 127: 64-66.

Tourtellotte, M.E. and M. Forsyth. (1988). Cloned *Mycoplasma gallisepticum* hemagglutinin gene product. In Compendium of abstracts of the7th International Congress of the IOM, Baden-Austria, Abstract No. P 20.

Trinder, P. (1969). Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J.Clin.Pathol., 22: 158-161.

Truscott, R.B., A.E.Ferguson, H.L.Ruhnke, J.R.Pettit, A.Robertson and G.Speckmann. (1974). An infection in chickens with a strain of *Mycoplasma gallisepticum* of low virulence. Can. J.Comp.Med., 38: 341-343.

Tully, J.G. (1983a). Cloning and filtration techniques for mycoplasmas. In: Methods in Mycoplasmology, Vol.I. Editors: S.Razin and J.G.Tully, Academic Press, New York. pp. 173-177.

Tully, J.G. (1983b). Dark-field microscopy. In: Methods in Mycoplasmology. Vol.I. Editors: S.Razin and J.G.Tully, Academic Press, New York. pp. 35-37.

Tully, J.G., R.F.Whitcomb, H.F.Clark and D.L.Williamson. (1977). Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. Sci., 195: 892-894.

Tully, J.G., D.Taylor-Robinson, D.L.Rose, R.M.Cole and J.Bove. (1983). *Mycoplasma genitalium*, a new species from the human urogenital tract. Int.J.Syst.Bacteriol., 33: 387-396.

Tully, J.G., D.L.Rose, R.E.McCoy, P.Carle, J.M.Bove, R.F.Whitcomb and W.G.Weisburg. (1990). *Mycoplasma melaleucae sp. nov.*, a sterol-requiring mollicute from flowers of several tropical plants. Int.J.Sys. Bact., 40: 143-147.

Turner, A.W. (1935). A study of the morphology and life cycles of the organism of pleuropneumonia contagiosa boum (*Borrelomyces peripneumoniae nov.* gen.) by observations in the living state under dark ground illumination. J.Path.Bact., 41: 1-32.

Uppal, P.K. and H.P.Chu. (1977). Attachment of *Mycoplasma gallisepticum* to the tracheal epithelium of fowls. Res.Vet.Sci., 22: 259-260.

Van Roekel, H. and O.M.Olesiuk. (1953). The etiology of chronic respiratory disease. Proc.90th AmVet.Med.Assoc., 289- 303.

Varley, J. and F.T.W.Jordan. (1978a). The response of chickens to experimental infection with strains of *M.gallisepticum* of different virulence and *M.gallinarum*. Avian Path., 7: 157-170.

Varley, J. and F.T.W.Jordan. (1978b). The response of turkey poults to experimental infection with strains of *M.gallisepticum* of different virulence and with *M.gallinarum*. Avian Path., 7: 383-395.

Wayne, C.L., M.Bennett, Y-S.Lu and S.P.Pakes. (1990). Biological evaluation of *Mycoplasma pulmonis* temperature-sensitive mutants for use as possible rodent vaccines. Infect.Immun., 58: 2289-2296.

Westerberg, S.C., C.B.Smith, B.B.Wiley, and C.Jensen. (1972). Mycoplasma-virus interrelationships in mouse tracheal organ cultures. Infect. Immun., 5: 840-846.

Weisburg, W.G., J.G.Tully, D.L.Rose, J.P.Petzel, H.Oyaizu, D.Yang, L.Mandelco, J.Sechrest, T.G.Lawrence, J.Van Etten, J.Maniloff and C.R.Woese. (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. J.Bact., 171: 6455-6467.

Whithear, K.G., Soeripto, K.G.Harringan and E.Ghiocas. (1990). Safety of temperature sensitive mutant *Mycoplasma gallisepticum* vaccine. Aust.Vet. J., 67: 159-165.

Wichmann, R.W. (1957). Case report: PPLO infection in chukar partridges (*Alectoris graeca*). Avian Dis., 1: 222-226.

Williams, C.O. and R.G.Wittler. (1971). Hydrolysis of esculin and phosphatase production by members of the order *Mycoplasmatales* which do not require sterol. Int.Syst.Bacteriol., 21: 73-77.

Williamson, D., J.G.Tully, D.L.Rose, K.J.Hackett, R.Henegar, P.Carle, J.M.Bove, D.E.Colflesh and R.F.Whitcomb. (1990). *Mycoplasma somnilux sp.nov., Mycoplasma luminosum sp. nov.*, and *Mycoplasma lucivorax sp. nov.*, new sterol-requiring *Mollicutes* from firefly beetles (*Coleoptera: Lampyridae*). Int.J.Sys.Bact., 40: 160-164.

Wills, F.K. (1955). Isolation of pleuropneumonia-like organisms from a peacock (*Pavo cristatus*). Southwest Vet., 8: 258-259.

Windsor, G.D., G.W.Thompson and N.W.Baker. (1975). Haemagglutination and haemagglutination inhibition with *Mycoplasma synoviae*. Res.Vet.Sci., 18: 59-63.

Woese, C.R., J.Maniloff and L.B.Zablen. (1980). Phylogenetic analysis of the mycoplasmas. Proc.Natl.Acad.Sci., 77: 494-498.

Yagihashi, T., T.Nunoya and M.Tajima. (1988). Pathogenicity for chickens of six strains of *Mycoplasma gallisepticum* isolated from various birds. Avian Path., 17: 725-729.

Yamada, S. and K.Matsuo. (1983). Experimental infection of ducks with *Mycoplasma gallisepticum.* Avian Dis., 27: 405-408.

Yamamoto, R. (1972). *Mycoplasma meleagridis* infection. In: Diseases of Poultry, 8th edition. Editors: M.S.Hofstad, B.W.Calnek, C.F.Helmboldt, W.M.Reid and H.W.Jr.Yoder. Iowa State University Press, Iowa, U.S.A. pp. 202.

Yamamoto, R. and H.E.Adler. (1958). Characterization of pleuropneumonia-like organisms of avian origin. II. Cultural, biochemical, morphological and further serological studies. J.Infect.Dis., 102: 243-250.

Yoder, H.W.Jr. (1986). A historical account of the diagnosis and characterization of strains of *Mycoplasma gallisepticum* of low virulence. Avian Dis., 30: 510-518.

Yoder, H.W.Jr. (1991). *Mycoplasma gallisepticum* infection. In: Diseases of Poultry, 9th edition. Editor: B.W.Calnek, Iowa State University Press, Iowa, U.S.A. pp. 198-212.

Yoder, H.W.Jr. and M.S.Hofstad. (1962). A previously unreported serotype of avian mycoplasma. Avian Dis., 6: 147-160.

Yoder, H.W.Jr. and M.S.Hofstad. (1964). Characterization of avian mycoplasma. Avian Dis., 8: 481-512.

Yogev, D., S.Levisohn, S.H.Kleven, D.Halachmi, and S. Razin. (1988a). Ribosomal RNA gene probes to detect intraspecies heterogeneity in *Mycoplasma gallisepticum* and *M synoviae.* Avian Dis , 32: 220-231

Yogev, D., S.Sela, H.Bercovier and S.Razin. (1988b). Elongation factor (EF-Tu) gene probe detects polymorphism in Mycoplasma strains. FEMS Microbiol. Letters, 50: 145-149.

Yogev, D., S.Levisohn and S.Razin. (1989). Genetic and antigenic relatedness between *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. Vet.Microbiol., 19: 75-84.

Zander, D.V. (1961). Origin of S6 strain mycoplasma. Avian Dis., 5: 154-156.

Zucker-Franklin, D., M.Davidson and L.Thomas. (1966). The interaction of mycoplasmas with mammalian cells. I. Hela cells, neutrophils, and eosinophils. J.Exp.Med., 124: 521-531.