Biofilm formation by mycoplasma species and its role in environmental persistence and survival

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Although mycoplasmas possess a very limited genome, little is known about their virulence mechanisms and methods of persistence in the host. Examination of a wide range of mycoplasma species found considerable variation in their ability to form a biofilm. *Mycoplasma putrefaciens*, *M. cottewii*, *M. yeatsii*, *M. agalactiae* and *M. bovis* produced prolific biofilms. Conversely, the highly pathogenic mycoplasma and causative agent of contagious bovine pleuropneumonia, *Mycoplasma mycoides* subsp. *mycoides* SC, was unable to produce a biofilm. Biofilms were found to be considerably more resistant to stress, including heat and desiccation, than planktonic cells. A link between the biofilm phenotype and genotype as determined by molecular typing was found for *M. bovis*. Analysis of biofilms using fluorescent staining combined with confocal microscopy demonstrated that mycoplasma biofilms formed a highly differentiated structure with stacks and channels. Biofilm formation may indicate that mycoplasmas are capable of surviving in the environment.

INTRODUCTION

Mycoplasmas are characterized by their small genome size and are thought to have undergone reductive evolution, losing many genes possessed by more complex bacteria in the process. They lack many genes, including those for cell wall synthesis and for the production of all 20 amino acids, as well as genes encoding enzymes of the citric acid cycle and the majority of all other biosynthetic genes (reviewed by Razin *et al.*, 1998). Presumably they can survive with a reduced genome as they have evolved in such a way as to acquire these products from their host *in vivo* and as such they are considered the model organisms for the study of essential functions in living cells. Despite their small genome size, mycoplasmas cause a wide range of disease in both humans and animals. They are associated with pneumonia, arthritis and reproductive disorders, and often cause disease of a chronic and persistent nature. However, very little is known about their pathogenicity factors or mechanisms of persistence in the host. There are only a few characterized virulence factors of mycoplasmas; these include, in certain mycoplasma species, the production of hydrogen peroxide (Brenann & Feinstein, 1969; Miles *et al.*, 1991), the carbohydrate capsule (Tajima *et al.*, 1982; Almeida & Rosenbusch, 1991), the ability to scavenge arginine from host cells (Sasaki *et al.*, 1984) and T-cell mitogens (Tu *et al.*, 2005). It is

difficult to explain how mycoplasmas manage to cause such severe and chronic infection given their paucity of virulence factors and lack of cell wall.

Bacteria are thought to persist in the host by the formation of an adherent biofilm. Biofilms can be defined as sessile bacteria attached to a substratum, or each other, often surrounded by an extracellular polysaccharide matrix. Biofilms often exhibit different phenotypes and physiology compared with planktonic cells, particularly with regard to gene transcription and growth rate (Donlan & Costerton, 2002). Perhaps the most important and widely studied property of biofilms is their vastly increased recalcitrance to host defences and resistance to stress, including that of antimicrobials: compared with planktonic cells, biofilms are commonly 10–1000 times more resistant (Mah & O'Toole, 2001; Costerton *et al.*, 1999). Unattached bacteria can be cleared by antibodies and phagocytes and are normally susceptible to antibiotics. However, adherent biofilm cells are resistant to antibiotics, antibodies and phagocytes. In addition, biofilms can cause host damage as phagocytes are attracted but phagocytosis is frustrated, and phagocytic enzymes are released which damage surrounding tissue and exacerbate infection. As well as enabling chronic infection of hosts, biofilms may cause bouts of acute infection when planktonic cells are periodically released from the biofilm. Despite their discovery in many other bacterial species studied to date, biofilms have not been studied in mycoplasmas. We

Abbreviation: CBPP, contagious bovine pleuropneumonia.

will investigate whether the phenomenon of biofilm formation is ubiquitous among bacterial genera and occurs even in organisms with a very limited genome. We propose that biofilm formation facilitates the survival of mycoplasma species in the environment and speculate whether it contributes to the persistence of mycoplasmas in the host.

METHODS

Strains and growth conditions. The bacterial strains used in this study are listed in Table 1. In addition, 56 *Mycoplasma bovis* strains as described in our previous study (McAuliffe *et al.*, 2004) were

Table 1. Mycoplasma strains used and their origins

Species	Strain	Origin
Mycoplasma agalactiae	NCTC 10123	Spain
Mycoplasma bovis	NCTC 10131	USA
Mycoplasma capricolum subsp. *capricolum*	California kid	USA
Mycoplasma mycoides subsp. *capri*	PG3	Unknown
Mycoplasma mycoides subsp. *mycoides* LC	Y goat	Australia
Mycoplasma mycoides subsp. *mycoides* SC	Afade	Chad
	KH3J	Sudan
	M375	Botswana
	N6	Botswana
	Tan8	Tanzania
	40F04	Namibia
	PG1	Kenya
	Gladysdale	Australia
	V5	Australia
	2091	France
	022	France
	138/5	Italy
	57/13	Italy
	197	Italy
	6526	Portugal
	410	Portugal
	6512	Portugal
	B103	Portugal
	6305	Portugal
	400	Portugal
	M545/91	Portugal
	Sauter	Spain
	Segonia	Spain
	Madrid	Spain
	Astleriae	Spain
Mycoplasma cottewii	NCTC 11732	Australia
Mycoplasma yeatsii	NCTC 11730	Unknown
Mycoplasma sp. bovine group 7	PG50	Australia
Mycoplasma putrefaciens	KS1	Unknown
Mycoplasma ovipneumoniae	NCTC 1051	Australia

used. All strains were stored at $-70\,^{\circ}$C. Planktonic cells were grown in Eaton's broth at $37\,^{\circ}$C with 5% CO_2 without aeration, as previously described (Nicholas & Baker, 1998). Biofilms were grown either on 11 mm^2 glass coverslips (Sigma) or in multiwell plates (Corning). Sterile coverslips were positioned vertically in a 50 ml conical tube (Corning) with 7·5 ml pre-warmed Eaton's medium. Coverslips were inoculated with a 1 : 100 dilution of a 20 h planktonic culture and left at $37\,^{\circ}$C with 5% CO_2 without aeration for 48 h. Biofilms were grown in multiwell plates by placing 180 µl medium in each well and inoculating with 20 µl of 20 h planktonic culture. Biofilms were left to grow for 48 h at $37\,^{\circ}$C with 5% CO_2 without aeration.

Semi-quantitative analysis of biofilm growth by crystal violet staining. Crystal violet staining was performed as described previously with minor modifications (O'Toole & Kolter, 1998). Biofilms grown on glass coverslips and in microtitre plates were rinsed briefly in PBS to remove non-adherent cells and stained with 0·5 % crystal violet solution for 30 min. Biofilms were then washed profusely (five times) in distilled water before being left to dry at room temperature for at least 30 min. Coverslips were broken into smaller pieces using sterile forceps and 1 ml 100 % ethanol was added to solubilize the crystal violet. Crystal violet in stained biofilms in microtitre plates was solubilized by the addition of 200 µl 100 % ethanol. Biofilm production was quantified by measuring the absorbance (560 nm) of 100 µl of the solubilized crystal violet in a microtitre plate. At least eight wells were analysed for each biofilm strain and each experiment was replicated in duplicate.

Assay of sensitivity of mycoplasma to stress of heat and drying. *M. bovis* biofilms were grown on glass coverslips for 48 h and subjected to stress of heat or drying. For the heat assay, biofilm and planktonic bacteria were exposed to a temperature of $50\,^{\circ}$C for 40 min. At 10 min intervals viable counts were made to enumerate the number of surviving bacteria, and triplicate samples were taken of both biofilm and planktonic cells. Coverslips were rinsed in PBS to remove non-adherent cells and were then placed into 2 ml Eaton's broth, broken into smaller pieces using sterile forceps, and vortexed for 1 min to remove biofilm cells from the coverslip. Viable bacteria were enumerated according to the methods of Miles & Misra (1938). Serial dilutions (20 µl) were performed in microtitre plates using 180 µl pre-warmed Eaton's medium as diluent. Triplicate aliquots of diluted cells (10 µl) were dropped on to over-dried Eaton's agar plates (without phenol red) from a height of approximately 1 cm. Plates were incubated at $37\,^{\circ}$C for 72 h and the resulting colonies counted.

For the drying assay, planktonic cells aliquoted on to 2 cm^2 pieces of sterile filter paper and biofilms on glass coverslips were placed in sterile Petri dishes and exposed to desiccation at room temperature for 31 h. Prior to the beginning of the assay, a piece of filter paper was placed briefly on top of the biofilms on coverslips to remove excess moisture. At intervals of 0, 3, 6, 17, 20, 24 and 31 h, triplicate biofilm and planktonic samples were taken and placed in 2 ml Eaton's medium, vortexed for 1 min and serial dilutions performed to enable the enumeration of surviving mycoplasmas as described above.

One-way analysis of variance was used to demonstrate significant differences between the performance of biofilm and planktonic cells in each assay.

Determination of effect of biofilm formation on the susceptibility to antibiotics for M. bovis and M. putrefaciens. *M. bovis* and *M. putrefaciens* biofilms and planktonic cells were subjected to various concentrations of the antibiotics danofloxacin, enrofloxacin and oxytetracycline. MICs were determined for planktonic cultures by diluting stationary-phase cultures to a standard OD_{600} of 0·005 in 10 ml Eaton's medium with appropriate

concentrations of antibiotics. Dilutions were made of the antibiotics to give a range of concentrations between 0·001 and 50 μg ml^{-1}. Planktonic cells were incubated with the antibiotic for 48 h and the tubes were examined for growth and colour change of the phenol red indicator. The MIC was determined as the lowest concentration of antibiotic that inhibited growth. Biofilms were grown on glass coverslips in 7·5 ml Eaton's medium for 24 h and then transferred to a fresh tube containing 10 ml Eaton's medium with the appropriate concentration of antibiotics. Biofilms were left in contact with antibiotic for 48 h and tubes were monitored for growth and colour change.

Confocal scanning laser microscopy. To examine the architecture of biofilm formation, *M. bovis* and *M. putrefaciens* were analysed by confocal microscopy. Biofilms were grown on glass coverslips and stained using the BacLight bacterial viability assay kit (Molecular Probes) according to the manufacturer's instructions. Essentially, live cells stained green with a SYT09 stain whereas dead cells with damaged membranes stained red with propidium iodide. Coverslips were mounted on to glass slides and examined using Perkin Elmer UltraView ERS using an excitation wavelength of 488 nm for SYTO9 and an emission filter of 500–550 nm; for propidium iodide an excitation of 568 nm and an emission filter of 580–650 nm were used. The presence of extracellular polysaccharide was also examined using Calcofluor White (Sigma) staining, with an excitation of 405 nm and an emission filter of 580–650 nm.

RESULTS

Biofilm formation by mycoplasma species

Eleven mycoplasma species were assessed for their ability to form a biofilm. At least two strains were examined for each species to ensure that there was no intraspecific variability in the ability to form a biofilm. Crystal violet staining of biofilms on coverslips revealed a striking variability in the ability of mycoplasma species to form a biofilm (Table 2). Some species, notably *M. putrefaciens*, *M. cottewii*, *M. yeatsii*, *M. bovis* and *M. agalactiae*, all formed prolific biofilms.

A clearly defined line of biofilm growth approximately 1–2 mm wide was seen on the coverslip at the air/liquid interface, and below this line the coverslip was heavily stained, indicating biofilm growth. *M. capricolum* subsp. *capricolum*, *Mycoplasma* sp. bovine group 7 and *M. ovipneumoniae* all showed very sparse biofilm formation with little staining evident. Interestingly, one species, *M. mycoides* subsp. *mycoides* SC, the causative agent of contagious bovine pleuropneumonia (CBPP), exhibited no biofilm formation at all. To confirm these results, an additional 24 strains of *M. mycoides* subsp. *mycoides* SC were analysed for their ability to form a biofilm and none was able to do so. Other mycoplasma species such as *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* LC showed an intermediate level of biofilm formation and the lower half of the coverslip below the air/liquid interface showed staining. Microscopic analysis of crystal-violet-stained *M. bovis* biofilms on coverslips indicated that individual cells were aggregating together to form microcolonies (Fig. 1). Biofilm growth by *M. bovis* was followed over a time-course of 48 h. By 20 h post-inoculation, cells had begun to adhere to the coverslips and a thin covering of individual cells could clearly be seen (Fig. 1a), by 36 h the cells had began to form small groups (Fig. 1b), and by 48 h larger groups of microcolonies were evident (Fig. 1c). Scanning electron microscopic (SEM) analysis of coverslips also indicated that whereas *M. bovis* formed a prolific biofilm, *M. mycoides* subsp. *mycoides* SC did not adhere very well. Coverslips were densely populated by *M. bovis* (Fig. 2a); conversely, only a few fields of view showed the presence of any *M. mycoides* subsp. *mycoides* SC at all (Fig. 2b).

Mycoplasmas were also analysed quantitatively for biofilm formation and the stationary-phase cell counts compared

Table 2. Ability of mycoplasma species to form a biofilm

Species	Qualitative crystal violet assay of biofilm formation*	Stationary-phase viable cell counts	
		Biofilm	Planktonic
M. agalactiae	+++	$6·2 \times 10^6$	$1·5 \times 10^8$
M. bovis	+++	$1·3 \times 10^7$	$1·6 \times 10^8$
Mycoplasma sp. bovine group 7	+	$2·8 \times 10^3$	$3·4 \times 10^9$
M. cotewii	+++	$4·6 \times 10^7$	$8·4 \times 10^8$
M. capricolum	+	$2·6 \times 10^3$	$2·5 \times 10^9$
M. ovipneumoniae	+	$1·8 \times 10^4$	$2·9 \times 10^8$
M. mycoides subsp. *mycoides* capri	++	$5·5 \times 10^5$	$1·8 \times 10^9$
M. mycoides subsp. *mycoides* LC	++	$6·7 \times 10^5$	$2·4 \times 10^9$
M. mycoides subsp. *mycoides* SC	−	$2·1 \times 10^1$	$9·5 \times 10^9$
M. putrefaciens	+++	$8·7 \times 10^6$	$5·8 \times 10^9$
M. yeatsii	+++	$3·8 \times 10^7$	$1·4 \times 10^9$

*−, no adherence; +, low-level adherence with only sparse staining and no line of biofilm formation at the air/liquid interface; ++, intermediate level of adherence with staining below the air/liquid interface but no defined line at the air/liquid interface; +++, high level of adherence with a clearly defined line of staining at the air/liquid interface and staining across the lower half of the coverslip.
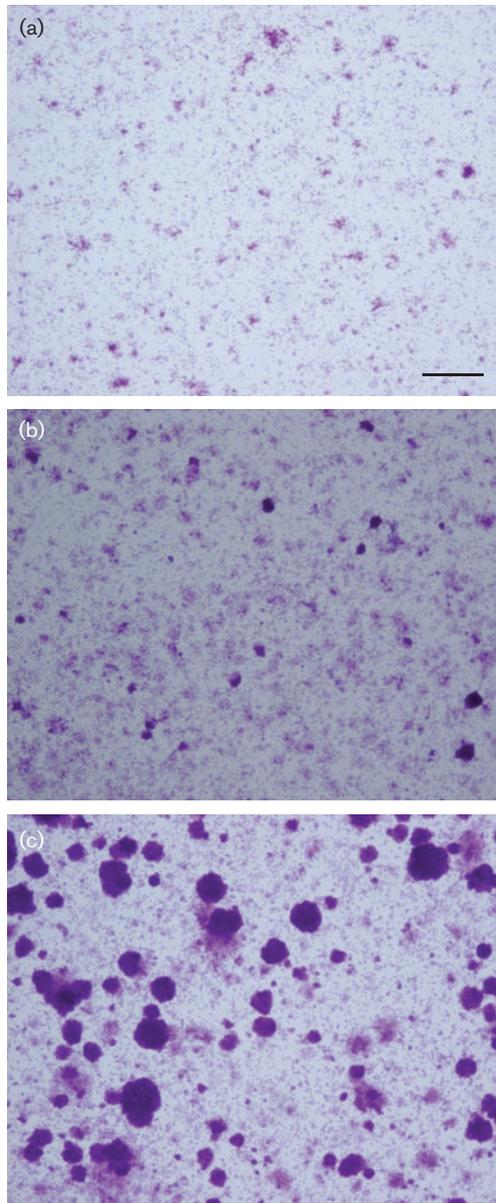
Fig. 1. Microscopic analysis of a biofilm formation on glass coverslips by *M. bovis* at (a) 20 h, (b) 36 h and (c) 48 h post-inoculation using crystal violet staining. Bar, 100 μm.

for both biofilm and planktonic cells. Cell counts for biofilms showed a good correlation with results obtained using crystal violet staining (Table 2). *M. putrefaciens*, *M. cottewii*, *M. yeatsii*, *M. bovis* and *M. agalactiae* demonstrated the most prolific biofilm formation, with cell counts in the range of 10^6–10^7 cells per coverslip, *M. mycoides* subsp. *mycoides* SC demonstrated minimal adherence (10^1 cells per coverslip) and the other species tested showed intermediate adherence (10^3–10^5 cells per coverslip).

M. mycoides subsp. *mycoides* SC strains were also tested on a variety of different surfaces to confirm that they were unable
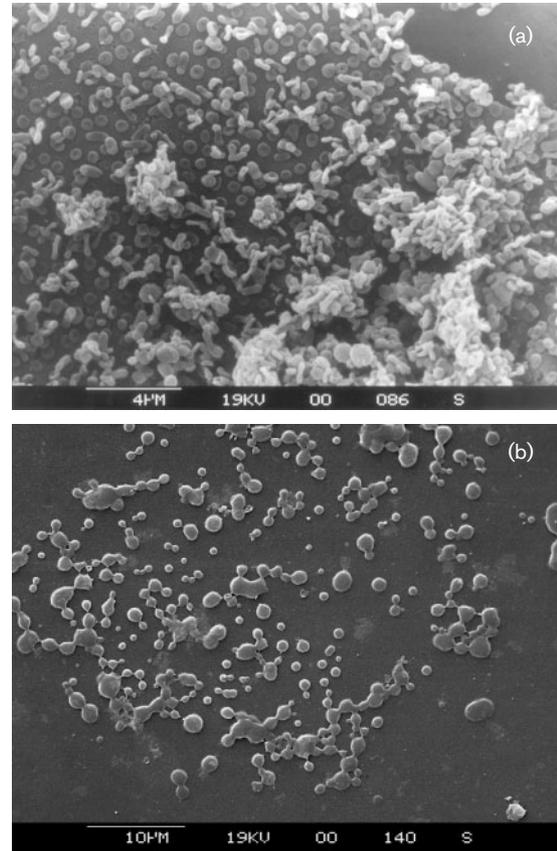

Fig. 2. SEM analysis of biofilm formation by *M. bovis* (a) and *M. mycoides* subsp. *mycoides* SC (b) cultures grown on glass coverslips.

to adhere. Tissue-culture-treated and untreated polystyrene multiwell plates (Corning), Nunclon tissue-culture-treated (Sigma) and polypropylene plates (Corning) were all tested. Tissue-culture-treated flasks, Nunc tissue-culture-treated 25 cm^2 and Corning 25 cm^2 cell culture flasks were also tested for SC adherence. However, *M. mycoides* subsp. *mycoides* SC was unable to adhere to any surface tested (results not shown).

Intraspecific variation in biofilm formation by *M. bovis* and correlation with molecular typing

M. bovis strains were found to vary widely in their ability to form a biofilm as determined by semi-quantitative crystal violet staining of biofilms grown on PVC microtitre plates (Fig. 3). Strains showing poor adhesion and biofilm formation gave A_{560} values as low as 0·08 whereas strains that formed prolific biofilms gave values up to 0·2. Interestingly, the type strain PG45 showed a low level of biofilm formation. There was some correlation between the ability to form a biofilm and the groups generated using molecular typing techniques (McAuliffe *et al.*, 2004), as shown in Table 3. Isolates that gave higher A_{560} values ($>0·15$) and formed more prolific biofilms, such as 158B01,
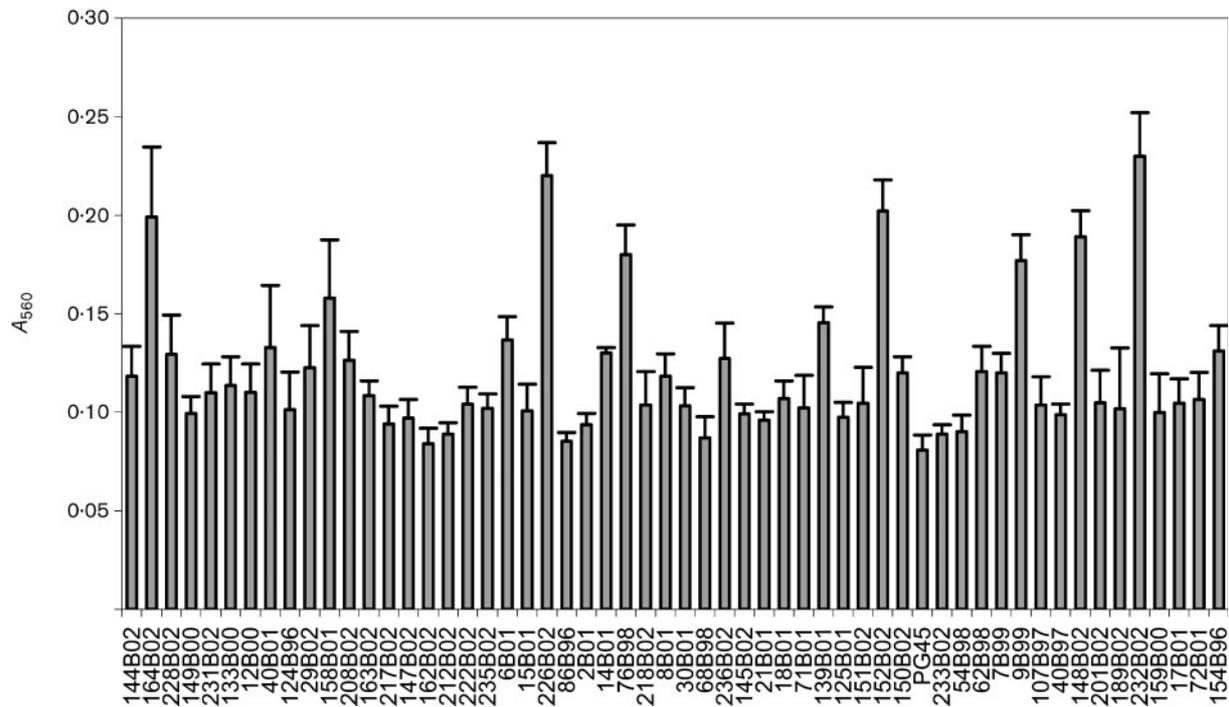
Fig. 3. Semi-quantitative analysis of *M. bovis* biofilm formation on microtitre plates using crystal violet staining. Isolates of *M. bovis* are derived from those described by McAuliffe *et al.* (2004).

76B98, 9B99, 164B02, 226B02, 152B01, 148B02 and 232B02, all fell into subgroup B as determined by amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD). Similarly, isolates that were unable to form an abundant biofilm (with A_{560} values <0·100), such as 147B02, 162B02, 68B98, PG45, 217B02,

Table 3. Influence of molecular typing profile and Vsp type on adherence of *M. bovis*

Strain	A_{560} of crystal-violet-stained biofilm	Molecular typing group as determined by AFLP (McAuliffe *et al.*, 2004)	Vsp profile as determined by Western blotting (McAuliffe *et al.*, 2004)
147B02	0·097	A8	BF
162B02	0·084	A5	BF
68B98	0·087	A5	F
PG45	0·080	A11	ABCO
217B02	0·094	A8	BF
86B96	0·085	A4	BF
125B01	0·097	A3	BF
54B98	0·090	A5	BF
2B01	0·093	A9	B
233B02	0·088	A4	BO
158B01	0·158	Bb1	O
76B98	0·182	Bb6	B
9B99	0·177	Ba11	BO
164B02	0·199	Ba10	B
226B02	0·223	Ba14	B
152B02	0·202	Ba12	BO
148B02	0·189	Ba15	BO
232B02	0·234	Bb4	BO

86B96, 125B01, 54B98 and 2B01, fell into typing group A using AFLP and RAPD.

When biofilm ability was compared with the Vsp profiles of the isolates (as determined in McAuliffe et al., 2004), it was found that there was a strong correlation between the expression of certain Vsps and biofilm formation (Table 3). The majority of isolates (70 %) that formed a poor biofilm (with A_{560} values less than 0·100) showed expression of VspF by Western blotting. Conversely, expression of VspF was not seen in any isolates that formed prolific biofilms (with A_{560} values in excess of 0·15) and expression of Vsps B and O was much more common, with VspB expressed in 86 % of isolates and VspO expressed in 63 % of isolates.

Contribution of biofilm formation to resistance of *M. bovis* to stress of heat and drying

Biofilm-grown *M. bovis* were more resistant to heat at 50 °C than planktonic *M. bovis* (Fig. 4a). For both strains analysed there was at least one log cycle more biofilm cells alive after 40 min compared with planktonic-grown cells. For strain 4, the 0·3 % biofilm cells alive was significantly greater ($P<0.0001$) than the 0·008 % planktonic cells alive after 40 min exposure to heat at 50 °C. Similarly, for strain 43, the 0·09 % biofilm cells alive was significantly greater ($P=0.022$) than the 0·002 % planktonic cells alive after 40 min at 50 °C.

Biofilm *M. bovis* were also more resistant to desiccation than planktonic-grown cells (Fig. 4b). There were no detectable surviving planktonic cells after 24 h drying whereas significantly greater proportions of strain 43 (0·0013 %, $P=0.009$) and strain 4 (0·01 %, $P=0.026$) biofilm cells were still viable after 32 h.

Effect of biofilm formation on the susceptibility of *M. bovis* and *M. putrefaciens* to antibiotics

Biofilm formation did not affect the MIC value of any of the antibiotics tested for either *M. bovis* or *M. putrefaciens*. Both planktonic and biofilm *M. bovis* strains 4 and 43 had an MIC of 0·75 µg ml^{-1} for danofloxacin, 25 µg ml^{-1} for oxytetracycline and 0·4 µg ml^{-1} for enrofloxacin. When *M. putrefaciens* was tested, it was found to have an MIC of 0·75 µg ml^{-1} for danofloxacin, 0·8 µg ml^{-1} for oxytetracycline and 0·4 µg ml^{-1} for enrofloxacin. Although the planktonic and biofilm MICs did not differ significantly, biofilm formation did have some phenotypic effects on *M. bovis*. It was found that exposure to oxytetracycline inhibited polysaccharide film formation in *M. bovis* at much lower concentrations of antibiotic for planktonic compared with biofilm cultures. Polysaccharide film production was prevented by 0·75 µg ml^{-1} in planktonic cultures but by 7·5 µg ml^{-1} in biofilm cultures.

Confocal microscopy of biofilms

Confocal imaging of 24, 48 and 72 h *M. bovis* and *M. putrefaciens* biofilms was undertaken. When biofilms were stained using a viability stain (BacLight, Molecular Probes) the majority of cells stained green at 24 and 48 h, indicating
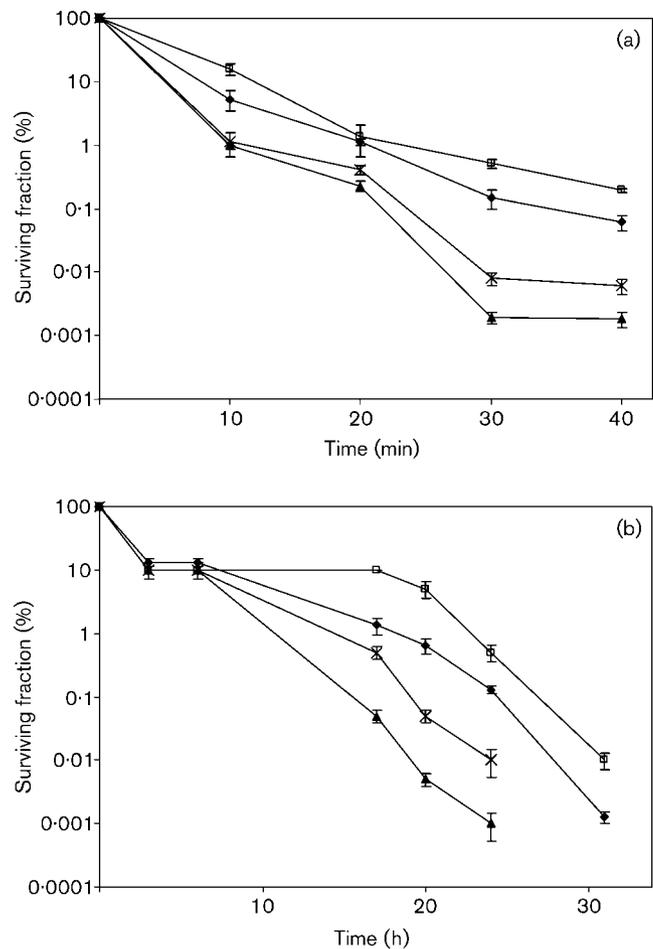

Fig. 4. Effect of heat at 50 °C (a), or desiccation (b), on survival of biofilm or planktonic cells of *M. bovis*. Biofilm grown strain 43 (◆), biofilm-grown strain 4 (□), planktonic strain 43 (▲), planktonic strain 4 (×). Bars indicate standard error ($n=3$).

that cells were live or had intact membranes. By 72 h the number of dead or membrane-compromised cells had increased to approximately 70 % of total cells, and live cells were seen predominantly in the centre of the biofilm (Fig. 5). Biofilms were seen to initiate from a layer of single, adherent cells at 24 h; by 48 h the biofilms had developed a more heterogeneous structure with stacks of cells approximately 20 µm high (Fig. 6). Channels were also seen between stacks. It was found that the SYTO9 stain stained both live and dead cells but as propidium iodide only stained dead cells these cells were easily differentiated by superimposing the images produced using the two wavelengths of excitation. Calcofluor white staining demonstrated a profuse layer of polysaccharide covering the biofilm (Fig. 7).

DISCUSSION

The mycoplasma species examined in this study demonstrated a remarkable diversity in their ability to form a
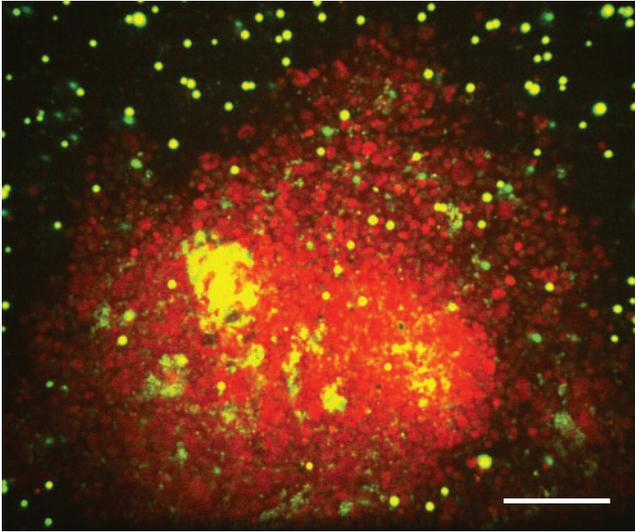
Fig. 5. Confocal image of *M. putrefaciens* biofilm with BacLight staining, showing persistence of live cells within microcolonies. Bar, 20 μm.

biofilm. Species such as *M. putrefaciens* and *M. bovis* formed a prolific biofilm that resembled that of other, more complex bacterial species. Biofilms were seen to form a highly differentiated structure with channels and stacks like those described in *Pseudomonas aeruginosa*, *Staphylococcus*
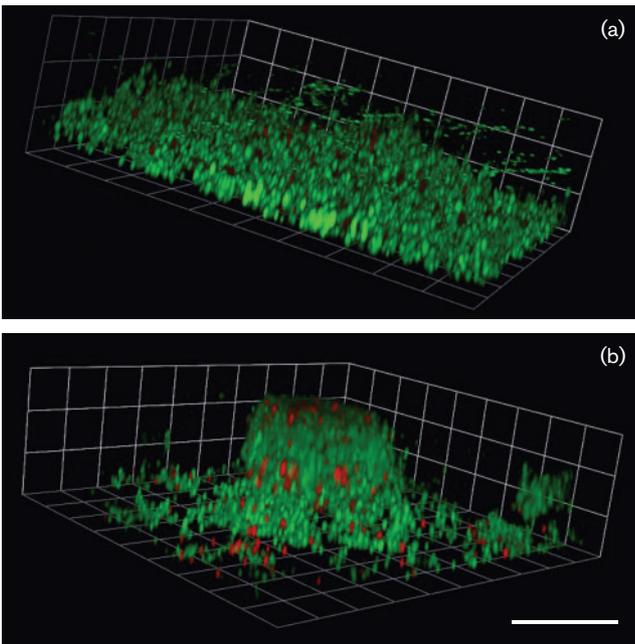

Fig. 6. Three-dimensional confocal image of *M. putrefaciens* biofilm stained using BacLight (Molecular Probes). After 24 h a layer of cells has formed on the coverslip (a); after 48 h stacks of cells can be seen within the biofilm (b). Bar, 20 μm.
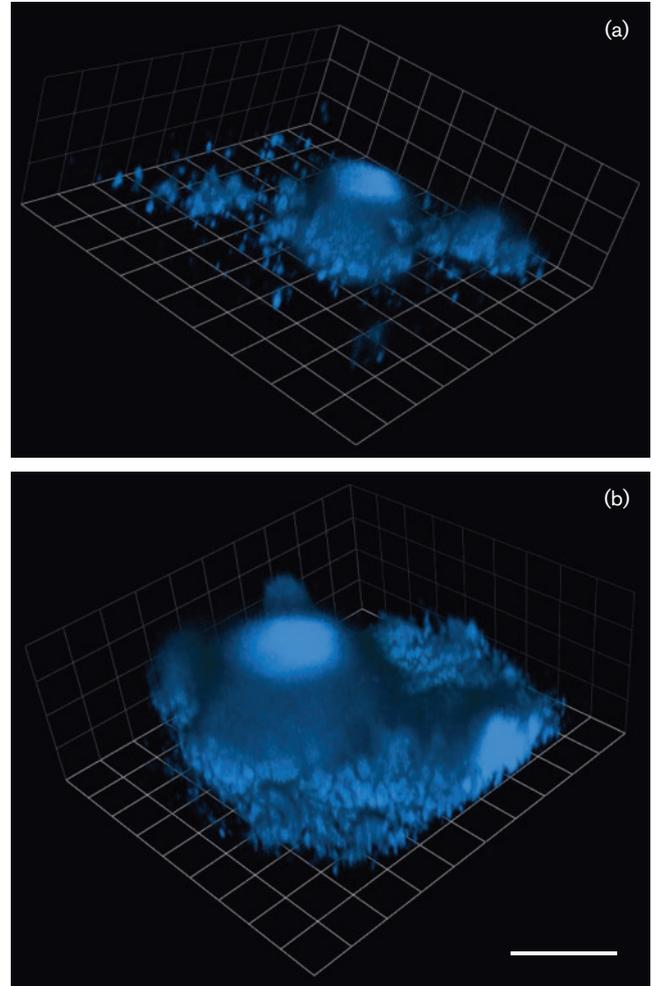

Fig. 7. Three-dimensional confocal image of calcofluor white-stained *M. putrefaciens* biofilms. A thick layer of polysaccharide (stained blue) can be seen covering stacks of cells within the biofilm Bar, 20 μm.

epidermidis and others (Dunne, 2002). Although many species were capable of forming a prolific biofilm, there was one notable exception, *M. mycoides* subsp. *mycoides* SC. Paradoxically, *M. mycoides* subsp. *mycoides* SC causes the most severe disease of all the mycoplasma species, with high mortality and morbidity, but it was unable to adhere to any surface tested in this study or to form a biofilm. Interestingly, the closely related small ruminant species *M. mycoides* subsp. *mycoides* LC did form a biofilm. Previous studies have also indicated that these two pathogens exhibit differing adherence abilities and reported that whereas LC adhered to erythrocytes and caprine endothelial cells, SC did not (Valdivieso-Garcia *et al.*, 1989). Intriguing studies by Minga (1981) described two colony types for SC. A smooth colony type was found that was thought to be more virulent, causing more widespread disease, with arthritis and weight loss in addition to respiratory symptoms. However, rough colony types caused more localized disease symptoms with

only respiratory involvement. Rough colony types were seen to form 'filaments and comets' in liquid media whereas smooth colony types gave a uniform turbidity. We speculate that the rough colony type may have been capable of biofilm formation and that the filaments and comets described by Minga were the first account of biofilm formation by a mycoplasma species. None of the 24 *M. mycoides* subsp. *mycoides* SC strains we tested showed rough colony morphology. It seems plausible that the inability of SC to adhere to the substrata tested in this study may be a result of the polysaccharide capsule produced by SC. The capsule of *M. mycoides* subsp. *mycoides* SC has been used as a diagnostic tool in a latex agglutination test (LAT) (March *et al.*, 2003). In the LAT, latex microspheres are coated with *M. mycoides* subsp. *mycoides* SC polyclonal immunoglobulin G antiserum and used to detect *M. mycoides* subsp. *mycoides* SC antigen in the serum of cattle infected with CBPP and in growth medium containing the organism (March *et al.*, 2003). It was found that there was cross-reactivity between *M. mycoides* subsp. *mycoides* SC, *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *mycoides* capri but not between other members of the mycoides cluster (March *et al.*, 2003). It is feasible that this is because these three mycoplasma species produce the galactan-based capsule and that other mycoides clusters produce a different capsule. We speculate that the galactan-based capsule of *M. mycoides* subsp. *mycoides* SC may inhibit adherence *in vitro*. Similarly, *M. ovipneumoniae*, which is also thought to produce a galactan-based capsule similar to SC (Niang *et al.*, 1998), was also unable to form a biofilm in this study. However, it has previously been suggested that the capsule may actually facilitate the adhesion of *M. ovipneumoniae* to the epithelium (Niang *et al.*, 1998). The idea that the polysaccharide capsule may actually inhibit adhesion has been proposed previously. In *Neisseria meningitidis*, an inverse correlation between capsule and biofilm formation has been described (Yi *et al.*, 2004). Similarly, in *Vibrio vulnificus*, capsule production has also been shown to prevent biofilm formation (Joseph & Wright, 2004). However, as many mycoplasma species such as *M. agalactiae* and *M. bovis* produce polysaccharide films, it will be essential to also examine the role of these macromolecules in biofilm formation.

This study is one of the first to address some fundamental questions regarding the survival of mycoplasma species when exposed to environmental stress. Previously it has been assumed that, given their paucity of resistance mechanisms and lack of cell wall, mycoplasmas would survive for very little time outside of laboratory culture or the animal host. Surprisingly, all the *M. bovis* isolates tested were much more resistant to heat than would be expected for an organism that lacks many of the heat-shock proteins commonly seen in other bacterial species. Mycoplasma species sequenced to date vary in the heat-shock-associated genes they possess. Most mycoplasma species do not possess GroE/L but *M. penetrans*, *M. pneumoniae* and *M. genitalium* do. The ClpB chaperonin is present in most mycoplasma species, with the exception of *M. gallisepticum* and *M. pulmonis*, but the ClpP/X proteases are not found in any sequenced mycoplasma species. Lon protease is also found in most mycoplasma species, as are the DnaK and DnaJ chaperonins.

Similarly, *M. bovis* was also surprisingly resistant to desiccation, with biofilm cells surviving over 30 h in the environment. As with heat shock, biofilm cells were significantly more resistant to drying than planktonic cells, providing further support for the notion that biofilm growth may help mycoplasmas to persist in the environment. Previously it has been shown in other bacterial species that biofilm cells are typically 10–1000 times more resistant to stress than their planktonic counterparts (Mah & O'Toole, 2001). The resistance of *M. bovis* to drying may have important implications for outbreak-handling and disease control, i.e. premises must be thoroughly disinfected following outbreaks and before restocking with cattle as it is plausible that *M. bovis* may persist in the environment. There is further evidence of the contribution of biofilm growth to the persistence of some mycoplasma species. When late-stationary-phase cultures that were starved of nutrients were observed using confocal microscopy and Live/Dead staining, it was found that live cells persisted in the centre of biofilm microcolonies even after all other cells around the edge of the biofilm had died. It seems likely that mycoplasmas in the centre of the biofilm are protected from environmental stress compared with free-living counterparts or those at the edge of the biofilm.

One of the most commonly studied properties of biofilms is their increased resistance to the effects of antibiotics and disinfectants. It is thought that the properties of biofilms that may cause recalcitrance to antibiotics include slow growth, phenotypic heterogeneity, the presence of persister cells, prevention of antibiotic penetration due to the exopolysaccharide matrix and inactivation of antibiotics within the biofilm matrix (Stewart, 2002). In this study, biofilm *M. bovis* was not found to be significantly more resistant to the actions of any of the antibiotics tested, although some antibiotics did cause phenotypic effects at concentrations below the MIC. The antibiotic oxytetracycline caused the inhibition of polysaccharide 'film' production in *M. bovis* planktonic cells at concentrations far below the MIC levels. However, film production was only inhibited by much higher concentrations of antibiotic in biofilm cells. Previous studies have also indicated that subinhibitory concentrations of antibiotics may have phenotypic effects, particularly on adhesion and polysaccharide production (Rachid *et al.*, 2000; Fonseca *et al.*, 2004).

This study has raised further questions regarding how mycoplasma species adhere to surfaces and initiate biofilm formation, as they lack all of the genes commonly associated with biofilm formation by other bacterial species. Mycoplasmas are thought to have evolved from the branch of low G+C Gram-positive species that includes the *Bacillus* and *Lactobacillus* species (Woese *et al.*, 2000).

They have undergone degenerative evolution, losing a large proportion of genomic DNA in the process (Woese *et al.*, 1980). It is therefore not surprising that they lack biofilm-associated gene homologues, such as *sigB*, that are normally present in Gram-positive organisms, or the staphylococcal genes *icaABC*, *bap* or its homologues *esp* and *bhp*, *arl* or *sarA*.

We also examined the influence of variable surface proteins on biofilm formation by *M. bovis*. Membrane lipoproteins and proteins are recognized virulence factors in mycoplasmas as they have been shown to induce blastogenesis and secretion of proinflammatory cytokines by a mechanism distinct from that of lipopolysaccharides (Brenner *et al.*, 1997; Herbelin *et al.*, 1994). In addition, membrane lipoproteins have been linked to biofilm formation and adhesion in *Escherichia coli* (Otto & Silhavy, 2002). There was found to be some correlation between the Vsp expressed by the strains and the ability to form a biofilm for *M. bovis*. Strains that expressed VspF showed poor biofilm formation whilst those expressing VspO or VspB formed prolific biofilms. These results are contradictory to the findings obtained using a bovine bronchial epithelial cell line, where the use of monoclonal antibodies to VspF significantly reduced the adherence of *M. bovis* (Thomas *et al.*, 2003). However, it is important to note that the monoclonal used in the study may show cross-reactivity against other surface lipoproteins and that it was not tested if the *M. bovis* strain used was expressing VspF (Thomas *et al.*, 2003). Previous studies have also shown a link between the expression of specific lipoproteins and adhesion for *M. bovis*. Sachse *et al.* (1996, 2000) demonstrated that the Vsp lipoproteins were involved in a variety of attachment and binding processes.

It is important to remember that this study has examined biofilm formation under *in vitro* laboratory conditions. Further studies utilizing animal models will be crucial to determine if biofilms form *in vivo* and whether they contribute to mycoplasma persistence in the host.

It has previously been suggested that as the inclination for bacteria to become surface bound is so ubiquitous in diverse ecosystems it suggests a strong survival and/or selective advantage for surface dwellers over their free-ranging counterparts (Dunne, 2002). We propose that the biofilm mode of growth offers a selective advantage in mycoplasmas, as it contributes to the persistence of many mycoplasma species. As mycoplasmas possess only a small genome, it seems likely that biofilm growth is beneficial, if not essential, for many mycoplasmas; otherwise it is likely that the genes necessary for this mode of growth would have been lost during degenerative evolution.

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