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1 ***Biofilm-grown Mycoplasma mycoides subsp. mycoides SC***
2 ***exhibit both phenotypic and genotypic variation compared with***
3 ***planktonic cells.***

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8

9 Running Title: Biofilm formation by *Mycoplasma mycoides* subsp. *mycoides* SC

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26

27

28 **Abstract**

29 Biofilm formation where bacterial cells adhere to a surface and surround themselves in a
30 polysaccharide matrix is thought to be an important factor in disease initiation and persistence
31 for many bacterial species. We have examined biofilm formation by *Mycoplasma mycoides*
32 subsp. *mycoides* small colony using a simple model without an air/ liquid interface and have
33 found that adherent *Mmm* SC was more resistant to many stresses, including heat, drying,
34 osmotic shock and oxidative stress. Biofilms of *Mmm* SC also exhibited remarkable persistence
35 and were able to survive for up to 20 weeks in stationary phase. Significant variation was seen
36 between *Mmm* SC strains in their ability to form a biofilm and the morphology of the biofilm
37 produced with some strains unable to produce microcolonies. Proteomic analysis found that a
38 number of proteins linked to adherence were over-expressed in biofilms compared with
39 planktonic cells.

40

41 **Introduction**

42 Contagious bovine pleuropneumonia (CBPP), an Office International des Epizooties (OIE) list A
43 disease is a severe respiratory disease of worldwide importance caused by *Mycoplasma*
44 *mycoides* subspecies *mycoides* small colony (*Mmm* SC). CBPP is currently absent from Europe
45 but which is now considered the most significant disease of cattle in Africa where it can cause
46 high morbidity and mortality.

47 *Mycoplasmas* possess small genomes and are thought to have evolved from more
48 complex bacteria by reductive evolution losing many genes in the process. They lack many
49 genes including those for cell wall synthesis, for the production of all 20 amino acids, as well as
50 genes encoding enzymes of the citric acid cycle and the majority of all other biosynthetic genes
51 (reviewed in Razin, Yogev and Naot, 1998). Currently, very little is known about the
52 pathogenesis of CBPP and of the persistence of *Mmm* SC in the host or the environment. Over
53 a century ago, when the cause of the disease was unknown, following severe outbreaks in the
54 USA there were indications that the organism may persist in the environment and it was written
55 that “ Many stables have been found in which the disease would appear and reappear after the

56 slaughter of affected herds, the destruction of the stable, the burning of the lumber, the removal
57 of the accumulations beneath the floors. It is conclusively shown that under some conditions
58 stables may retain the infection for a considerable time and that when restocked disease may
59 break out again." (Salmon 1896). It has never been explained how mycoplasmas that are
60 seemingly so fragile and lacking a cell wall could survive in the environment.

61 In many other bacterial species adherence to a solid surface and biofilm formation are important
62 steps in the initiation of disease. Previously, we have demonstrated that some *Mycoplasma*
63 species form biofilms and have speculated that this may enable them to persist in the
64 environment and may even contribute to disease in the host (McAuliffe *et al.*, 2006).

65 Mycoplasma biofilms were found to begin by a thin covering of individual cells followed by the
66 formation of small groups of cells and after prolonged incubation larger groups of microcolonies
67 that gave a characteristic stack-like structure with channels between stacks as seen in other
68 bacterial biofilms were evident (McAuliffe *et al.*, 2006). Although *Mycoplasma* species including
69 *M. bovis*, *M. putrefaciens*, *M. cottewii* and *M. agalactiae* formed prolific biofilms and were more
70 resistant to stresses including heat and desiccation when grown as a biofilm, we found that
71 *Mmm* SC would not adhere to any of the surfaces we tested when an air/liquid interface model
72 was used and cells were found to exist in a free-living state rather than adhere to a substratum
73 (McAuliffe *et al.*, 2006). However, in this study we will demonstrate that *Mmm* SC can be studied
74 as an adherent layer when it is grown on a membrane placed on an agar plate in a simple
75 biofilm model that lacks an air/liquid interface.

76 In this study we examined *Mmm* SC grown on a solid medium and compared it with cells
77 grown in identical liquid medium. We have found that *Mmm* SC is not only more resistant but
78 exhibits differential gene expression when attached to a solid surface, and propose that surface
79 adherence may be an important process in disease initiation by *Mmm* SC.

80 Fundamental studies of this type have rarely been undertaken in mycoplasmas but perhaps
81 because of their lack of cell wall and paucity of resistance mechanisms it was always thought
82 they would not survive outside of the animal host.

83

84 **Materials and methods**

85 **Strains and growth conditions**

86 The bacterial strains used in this study are listed in table 1. All strains were stored at -70°C in
87 Eaton's broth. Planktonic cells were grown in Eaton's broth as previously described for 18 h at
88 37°C with 5 % CO_2 without aeration (Nicholas and Baker, 1998). Adherent cells were grown on
89 $0.1\ \mu\text{m}$ pore size, 48 mm diameter membranes cellulose acetate filter membranes (Sartorius)
90 on Eaton's agar plates (without phenol red indicator) for 48 h at 37°C with 5 % CO_2 without
91 aeration. All cells examined were in stationary phase.

92

93 **Variation in biofilm formation among *Mmm* SC strains.**

94 18 *Mmm* SC strains were analyzed for the ability to form a biofilm. Stationary phase planktonic
95 cultures were diluted 1:50 and 50 μl aliquots were dropped onto filter membranes placed on
96 Eaton's agar plates without phenol red. The membranes were aseptically cut into 4 equal sized
97 segments prior to inoculation. Filter membranes on plates were incubated for 72 h with duplicate
98 membranes taken at 8 and 24 h intervals to monitor growth. Membranes were placed into 1 ml
99 of Eaton's medium in a 30 ml screw-topped tube, adherent cells were removed from the
100 membranes by vortexing for 1 min and were then enumerated by performing serial dilutions in
101 microtitre plates, taking 100 μl aliquots and plating onto Eaton's agar plates and counting the
102 resulting colonies after 48 h incubation. Table 1 shows the highest stationary phase cell
103 densities recorded.

104

105 **Heat shock and desiccation assay**

106 *Mmm* SC strains B103 and Afadé were grown either as planktonic cultures in broth or as
107 adherent cells on membranes on agar plates for 48 h and subjected to stress of heat or drying.
108 For the heat assay adherent and planktonic bacteria were exposed to 53°C for 50 min.
109 Adherent cells on membranes were placed in a 30 ml screw-topped tube (Sterilin) with 2 ml of
110 prewarmed Eaton's broth, planktonic cells with added to a sterlin tube with 2 ml of prewarmed
111 Eaton's broth to give a final density of 10^6 cfu/ml. At 10 min intervals, viable counts were made
112 to enumerate the number of surviving bacteria; triplicate samples were taken of both adherent
113 and planktonic cells. Membranes were aseptically removed and placed in a fresh tube containing
114 1 ml of Eaton's broth held at room temperature. A 200 μl aliquot of the heated planktonic cells
115 was placed in a fresh tube with 800 μl Eaton's broth at room temperature. Tubes containing

116 membranes and planktonic cells were then vortexed for 1 min and viable bacteria were
117 enumerated. Serial dilutions (20 μ l) were performed in microtitre plates using 180 μ l of 37 °C
118 Eaton's medium as diluent. Triplicate aliquots of diluted cells (100 μ l) were spread onto Eaton's
119 agar plates (without phenol red). Plates were incubated at 37 °C for 72 h and the resulting
120 colonies counted.

121

122 For the desiccation assay biofilms grown on quartered membranes and planktonic cells of
123 approximately the same density were subjected to drying. Planktonic cells (10 μ l) were
124 aliquoted onto 2cm² pieces of sterile filter. Planktonic cells on filter paper sections and biofilms
125 on membranes were placed in sterile petri dishes and exposed to desiccation at 20 °C for 31 h.
126 At intervals of 0, 1, 2, 3, 4, 5, and 25 h, triplicate biofilm membrane sections and planktonic filter
127 paper sections were taken and placed in 2 ml of Eaton's medium, vortexed for 1 min and serial
128 dilutions performed to enable the enumeration of surviving mycoplasmas as described above.

129

130 **Osmotic shock, oxidative stress and detergent stress assays**

131 Stationary phase *Mmm* SC grown either as planktonic cultures in broth or as adherent cells on
132 membranes on agar plates were subjected to stress of 5M NaCl or 100 mM H₂O₂ or 0.005%
133 sodium dodecylsulphate (SDS) for 50 min. Adherent cells were placed in a 30ml screw-topped
134 tube (Sterilin) with 2 ml of stressor solution, while planktonic cells were added to a 30ml screw-
135 topped tube (Sterilin) with 2 ml of the same solution to give a density of 10⁶ cfu/ml. Triplicate
136 samples were taken of both adherent and planktonic cells at 10 min to enumerate the number of
137 surviving bacteria. Membranes or a 200 μ l aliquot of the planktonic cells were aseptically
138 removed from each treatment tubes and placed in a fresh tube containing volumes of with 1 ml
139 and 800 μ l of Eaton's broth respectively. Tubes containing membranes and planktonic cells were
140 then vortexed for 1 min, and viable bacteria were enumerated as described above.

141

142 **Long term survival assay**

143 The survival of 4 strains of *Mmm* SC in stationary phase culture over a period of 20 weeks at 20
144 °C was monitored. At 24 h intervals, an aliquot was removed and the number of viable cells

145 remaining determined by serial dilution as described above.

146

147 **Confocal microscopy**

148 *Mmm* SC biofilms were analysed by confocal laser microscopy. Biofilms were grown on
149 membranes on Eaton's agar plates were stained using the BacLight bacterial viability assay kit
150 according to the manufacturers instructions (Molecular Probes) live cells stain green with a
151 SYTO9 stain whereas dead cells with damaged membranes stain red with propidium iodide.
152 Membranes were mounted onto glass slides and examined with a Perkin Elmer UltraView ERS
153 spinning disk confocal microscope. For SYTO9, an excitation wavelength of 488 nm and an
154 emission filter of 500-550 nm was used, while for propidium iodide, an excitation of 568 nm and
155 an emission filter of 580-650 nm was used.

156

157 **SDS PAGE and protein identification**

158 Whole cell proteins were collected from 2 *Mmm* SC strains, Afadé and B103. Planktonic cultures
159 (20 ml) and adherent cells grown on membranes on Eaton's plates and separated by 1D and 2D
160 SDS-PAGE. Adherent cells were harvested by vortexing membranes in 4 °C (5 ml) phosphate
161 buffered saline (PBS). Planktonic cells and adherent cells in PBS were pelleted by
162 centrifugation at 6,000 x g at 4°C. Cell pellets were washed thrice with PBS, resuspended in
163 100 µl of distilled water and protein estimations were carried out using a BCA assay kit (Pierce).
164 Protein concentrations were normalised to give 1 mg/ml. For 1D SDS PAGE, proteins were
165 boiled in SDS PAGE sample buffer for 10 mins and for 2D analysis proteins were then
166 precipitated (Biorad ReadyPrep 2D clean up kit). For 1D SDS PAGE, samples were run on 12
167 % SDS PAGE gels according to the methods of Laemmli (Laemmli, 1970). For 2D SDS PAGE
168 the samples (approximately 400 µg of protein) were air dried for 4 minutes and resuspended in
169 300 µl of isoelectric focusing sample buffer (Biorad). The protein samples were separated by
170 using immobilized pH gradient strips (Biorad) in the pH range from 4 to 7. Strips were then
171 separated for the second dimension on 10 % acrylamide gels for 16 h at 75 V and all gels were
172 stained with Coomassie blue for 1h and destained in 40 % methanol/10 % acetic acid for 8 h.
173 Any bands which showed differential expression in either biofilm or planktonic cells were excised

174 using a sterile scalpel blade, and proteins were identified using ESI-MS/MS sequencing
175 (Eurogentec, Belgium).

176 **Results**

177 **1. Strain variation in ability of *Mmm* SC to form an adherent cell layer.**

178 *Mmm* SC strains varied considerably in their ability to grow as an adherent layer on a membrane
179 on an Eaton's agar plate. Final stationary phase densities varied between 4×10^5 and 1×10^9 cfu
180 per membrane (table 1). There were also differences in biofilm morphology between the strains
181 examined, Afadé, N6 and Tan8 grew very profusely and demonstrated an unusual morphology
182 with large micro-colonies or stack-like structures with many cells deeply interspersed amongst
183 the flatter layer of cells which covered the membrane (Figure 1). Strain V5 showed a densely
184 packed, profuse biofilm with smaller stacks than Afadé and very little space between stacks. It
185 was unusually covered in a thick polysaccharide material containing DNA which was detected
186 with the nucleic acid stain (Figure 2A). Some strains such as 2091, O526 and IS31 produced a
187 flatter biofilm which was still several cells deep, and with microcolonies around the edge of the
188 biofilm . Other European strains such as 138/5, B103, 197, Madrid and Segovia all showed poor
189 cell growth on membranes. Strains 138/5 and B103 exhibited the poorest growth of all with only
190 a few adherent cells and no stacks/micro-colonies of cell aggregates seen at all (Figure 2B).
191 Strains 197, Madrid and Segovia showed sparse biofilm formation with a very thin monolayer .

192

193 **2. Effect of biofilm formation on the resistance of *Mmm* SC to stress**

194 Adherent *Mmm* SC were found to be significantly more resistant to heat at 53 °C with both Afadé
195 and B103 strains at least one log cycle more resistant than the corresponding planktonic cells
196 (figure 3A). When *Mmm* SC was exposed to desiccation at 20 °C some variation in susceptibility
197 was seen according to strain and mode of growth. Overall Afadé cells were demonstrated to be
198 the most resistance to drying with biofilm cells surviving for 25 hours and all cells, except
199 adherent Afadé, were killed by 25 hours (figure 3B).

200 Mycoplasmas showed remarkable resistance to osmotic shock when tested using 5M
201 NaCl as there only limited killing; less than one log reduction was seen in both planktonic and
202 adherent cells and for the two strains tested (results not shown).

203 Biofilm *Mmm* SC were found to be considerably more resistant to exposure to 100mM
204 peroxide than planktonic cells. There were no survivors detected for either planktonic B103 or
205 Afadé after 20 min exposure however biofilm grown Afadé and B103 had 2% survivors at 20
206 minutes. No survivors were detected for any of the strains tested after 30 minutes exposure
207 (figure 4A).

208 Biofilm grown *Mmm* SC were significantly more resistant to killing with 0.05% SDS
209 compared with planktonic cells. Less than one log reduction was seen with adherent Afadé and
210 B103 yet the respective planktonic cells showed over 4 log reduction after 40 min exposure to
211 SDS (figure 4B).

212 Long term survival studies of stationary phase adherent cells showed a considerable
213 variation in the persistence of strains. Strain B103 was the least persistent with only 10^2
214 cfu/membrane detected after 2 weeks and no surviving cells detected after 5 weeks. Strain N6
215 gave detectable surviving cells (10^1 cfu/membrane) after 5 weeks but no survivors after 6 weeks.
216 Strain 2091 had detectable survivors after 10 weeks (10^2 cfu/membrane) and stain Afadé had
217 detectable surviving cells (10^2 cfu/membrane) even after 20 weeks in stationary phase.

218 3. Changes in protein profile induced by adherence

219 Whole cell proteins were analysed using both 1D (Figure 5) and 2D SDS PAGE and ESI-MS/MS
220 for planktonic and biofilm *Mmm* SC strains Afadé and B103. Differences in protein profile
221 between the strains were determined using Bionumerics 2D and any spots/bands which differed
222 between biofilms and planktonic cells were excised and identified using EMI MS/MS
223 (Eurogentec). Several proteins which showed differential expression under conditions of biofilm
224 growth were identified using ESI-MS/MS sequencing. Proteins highly expressed in biofilm cells
225 but to a lesser extent in planktonic cells were found to match elongation factor Tu of *M. c.*
226 *capricolum* (Q2SSW8_MYCCT), pyruvate dehydrogenase (lipoamide) alpha chain
227 (Q6MTY0_MYCMS), PTS system glucose-specific IIBC component (Q6MSA5_MYCMS),
228 phosphoenolpyruvate-protein phosphotransferase (Q6MTX2_MYCMS), fructose-bisphosphate
229 aldolase class II (Q6MU98_MYCMS) and hypoxanthine phosphoribosyltransferase of *M. c.*

230 *capricolum* (Q2SSR0_MYCCT). Afadé biofilms also preferentially expressed a protein with
231 similarity to triosephosphate isomerase (Q6MSF2_MYCMS) .

232

233

234 **Discussion**

235 Previously we have found that although many *Mycoplasma* species form prolific biofilms, *Mmm*
236 SC did not when analysed using a glass coverslip model with an air/liquid interface. In order to
237 examine more closely the behaviour of *Mmm* SC when attached to a surface we have
238 developed a biofilm model system which does not have an air liquid interface and in which *Mmm*
239 SC can be grown as an adherent layer and compared with planktonic cells.

240 Bacterial biofilms are considered to be 10-1000 times more resistant than planktonic
241 cells to all stress (Mah and O' Toole, 2001; Costerton, Stewart and Greenberg, 1999). Overall,
242 adherent *Mmm* SC cells were significantly more resistant to the stresses they were exposed to
243 during this study. Biofilm-grown *Mmm* SC were more resistant to peroxide and detergent
244 stress, and heat stress than planktonic cells. These results are not surprising as for example,
245 although not previously studied in *Mycoplasma*, resistance to hydrogen peroxide has been
246 widely studied in other bacterial species where it has been shown that biofilms are considerably
247 more resistant than planktonic cells (Elkins *et al.*, 1999, Wen *et al.*, 2005). Oxidative stress
248 resistance is one of the key properties that enables pathogenic bacteria to survive the effects of
249 reactive oxygen species produced by host cells (Storz and Zheng, 2000). Intriguingly, the
250 production of hydrogen peroxide is also thought to be an important virulence factor for *Mmm* SC
251 itself (Miles *et al.*, 1991) but it remains unclear why the high levels of H₂O₂ that must be
252 produced to act as a virulence factor are not toxic to the mycoplasmal cell. Mycoplasmas do not
253 possess most of the enzymes that normally contribute to oxidative stress resistance in other
254 bacterial species, such as catalase, and *Mmm* SC lacks most of the SoxR/SoxS oxidative shock
255 regulon with the exception of the endonuclease IV *nfo* and the iron regulator *fur*. In addition,
256 *Mmm* SC completely lacks the OxyR regulon that is crucial for oxidative stress resistance in
257 many other bacterial species and even lacks a homolog to MsrA which protects against oxidative

258 damage in *M. genitalium* (Dhandayuthapani *et al.*, 2001). Thioredoxin reductase is thought to
259 act as a detoxifying system protecting mycoplasmas from oxidative damage by reactive oxygen
260 species (ROS) and this system has been found in every mycoplasmal species tested to date,
261 including *Mmm* SC (Ben-Menachem *et al.*, 1997). Our study shows that *Mmm* SC is remarkably
262 tolerant to peroxide and that biofilm formation contributes to its resistance. We found the African
263 strain Afadé was more resistant compared with European strain B103; these results are
264 intriguing as it is also thought that African *Mmm* SC strains may produce more hydrogen
265 peroxide than European strains because isolates from recent European outbreaks have lacked
266 part of the glycerol uptake genes due to a deletion (Pilo *et al.*, 2005).

267 Perhaps one of the most important findings of this study relates to the persistence of
268 *Mmm* SC: one of four strains examined was found to survive for in excess of 20 weeks when
269 grown as an adherent biofilm. Obviously this finding may have implications for outbreak handling
270 and control as it has previously been assumed that *Mmm* SC, and other *Mycoplasma* species
271 would not survive in the environment. Further work is necessary to determine whether *Mmm* SC
272 can survive under true environmental conditions on surfaces likely to be encountered on farms,
273 such as straw and wood.

274 Significant differences were seen between the Afadé African strain of *Mmm* SC and
275 European strain *Mmm* SC B103 biofilms, both in terms of morphology of the biofilm and its
276 resistance to some stresses. Previous studies by many authors have indicated that African
277 strains result in higher morbidity and more severe pathology than European strains during CBPP
278 infections. Recent European infections have been typically chronic and insidious in nature, with
279 cattle exhibiting few clinical signs and rarely dying (Nicholas & Bashiruddin, 1995 genetic
280 differences have been suggested for the differing mortality and pathology patterns seen between
281 infections with African and European strains of *Mmm* SC (Nicholas & Bashiruddin, 1995). It is
282 known that European strains of *Mmm* SC lack 8.8KB of genomic DNA which is present in African
283 strains (Vilei *et al.*, 2000). This segment contains a copy of IS1634, a gene for a potential
284 lipoprotein, LppB, a putative surface-located membrane protein and a hypothetical proline-rich
285 membrane protein, and two open reading frames showing similarity to putative ABC
286 transporters. Other genetic differences between African and European strains include PtsG, a

287 glucose phosphotransferase system permease, where European strains have a single copy of
288 the *ptsG* gene but non-European *Mmm* SC strains have two copies (Gaurivaud *et al.*, 2004).

289 It is often thought that programmed changes in gene expression results in a specific
290 biofilm phenotype that is more resistant to stress and exhibits differential gene expression
291 compared with planktonic cells. We have identified a number of proteins that appear to be
292 specifically expressed in biofilms but not in planktonic cells although the genetic basis of biofilm
293 formation is yet to be fully elucidated. Mycoplasmas possess none of the known regulatory
294 systems that are involved in biofilm formation in other bacterial species; *rpoS* in gram negative
295 bacteria such as *Escherichia coli* (Prigent-Combaret *et al.*, 2001, Corona-Izquierdo and
296 Membrillo-Hernandez, 2002), *SpoA* of *Bacillus subtilis* (Hamon and Lazazzera 2001), *sarA* of
297 *Staphylococcus epidermidis* (Valle *et al.*, 2003) or *sinR* of *Bacillus subtilis* (Kearns *et al.*, 2005).
298 As mycoplasmas possess only a single sigma factor and no known master regulators it is
299 unlikely that biofilm formation could be regulated by a complex regulatory system similar to those
300 seen in higher bacterial species. Recently it has been shown in *M. pulmonis* that biofilm
301 formation occurs stochastically as a result of rearrangements in variable surface antigens and is
302 not due to complex changes in programmed gene expression (Simmons *et al.*, 2007). We have
303 yet to discover if a similar system exists in *Mmm* SC but we have previously reported that biofilm
304 formation in *M. bovis* there was some correlation between variable surface protein expression
305 and ability to form a biofilm (McAuliffe *et al.*, 2006). It has been well documented that *Mmm* SC
306 possesses various variable surface antigens (Westburg *et al.*, 2004) and these may possibly
307 influence biofilm formation in a similar manner.

308 In this study proteomic analysis revealed several proteins that were up-regulated when
309 *Mmm* SC was grown as an adherent biofilm; elongation factor Tu, the PTS system glucose-
310 specific transporter IIB component, phosphoenolpyruvate protein phosphotransferase, fructose-
311 bisphosphate aldolase class II, triosephosphate isomerase and pyruvate dehydrogenase .
312 Interestingly pyruvate dehydrogenase and elongation factor Tu are thought to play a role in the
313 binding of *M. pneumoniae* to the extracellular matrix component fibronectin (Dallo *et al.*, 2002)
314 and are also thought to be important parts of the cytoskeleton of *M. pneumoniae* and as such,
315 are linked to cell adhesion (Layh-Schmitt *et al.*, 2000). PTS system proteins and Tu have also
316 been linked to stress response in *M. pulmonis* (Fehri *et al.* 2005). Other proteins identified such

317 as fructose-bisphosphate aldolase and triosephosphate isomerase are involved in carbohydrate
318 catabolism. A recent study of protein expression in *Streptococcus mutans* biofilms showed that
319 glycolytic enzymes analogous to the ones found in this study were heavily expressed during
320 early biofilm formation (Welin et al 2004). Therefore it seems feasible that some glycolytic
321 enzymes are not simply limited to substrate turnover and may also have functions in other
322 processes, including biofilm formation.

323 Biofilms generally have a heterogeneous nature with cells exposed to gradients of
324 nutrients and oxygen, and generally exhibit a slower growth rate, both of which may make cells
325 inherently more resistant to many stresses (Mah and O'Toole, 2001). Additionally, they have a
326 protective polysaccharide layer that delays the penetration of chemical agents such as
327 antibiotics and ROS. Therefore, in addition to the induction of genes which may lead to
328 increased resistance to stress, mycoplasma biofilms may also be intrinsically more resilient as
329 the physicochemical properties and architecture of biofilms can engender resistance to stress in
330 resident cells.

331

332

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336

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432 *mycoides* SC type strain PG1T, the causative agent of contagious bovine pleuropneumonia
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434

435

436 Table 1. Variation in biofilm formation among *Mmm* SC strains in stationary phase

| Strain | Country and year of origin | Density of adherent cells/membrane | Density of planktonic cells/ml |
|------------|----------------------------|------------------------------------|--------------------------------|
| V5 | Australia 1936 | 1×10^9 | 4×10^8 |
| 57/13 | Italy 1992 | 9×10^8 | 5×10^8 |
| Afadé | Chad 1968 | 7×10^8 | 9×10^8 |
| Tan8 | Tanzania 1998 | 5×10^8 | 2×10^8 |
| IS31 | Tanzania 1998 | 4×10^8 | 2×10^9 |
| 2022 | France 1984 | 3×10^8 | 5×10^8 |
| O526 | Portugal 1993 | 2×10^8 | 7×10^8 |
| C305 | Portugal 1993 | 1×10^8 | 3×10^8 |
| B103 | Portugal 1986 | 1×10^8 | 9×10^7 |
| M545/91 | Portugal 1991 | 8×10^7 | 1×10^9 |
| Gladysdale | Australia Pre-1964 | 5×10^7 | 1×10^9 |
| N6 | Botswana 1996 | 5×10^7 | 1×10^8 |
| 197 | Italy 1992 | 2×10^7 | 3×10^8 |
| Segovia | Spain 1991 | 2×10^6 | 6×10^8 |
| 2091 | France 1984 | 2×10^6 | 3×10^8 |
| Santander | Spain (Year unknown) | 1×10^6 | 6×10^8 |
| 138/5 | Italy 1992 | 8×10^5 | 1×10^8 |
| Madrid | Spain 1984 | 14×10^5 | 2×10^8 |

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442 Figure legends

443 Figure 1. Confocal laser imaging of 72 h adherent *Mmm* SC strain Afadé stained using BacLight
444 live/dead staining. A large aggregate of cells forming a circular stack up to 30 μm in height can
445 be seen, cells around the outside of the stack are dead (stained red) cells whereas within the
446 centre of the stack cells are able to persist (stained green) (A). A flat monolayer of cells less than
447 10 μm high can be seen in channels between the stacks (B).

448

449 Figure 2. Confocal laser imaging of 72 h adherent *Mmm* SC strain V5 showing a prolific biofilm
450 with a dense covering of extracellular material (A) and strain B103 showing very sparse biofilm
451 formation with only a few small cells attached to the surface (B).

452

453

454 Figure 3. Effect of biofilm growth on the resistance of *Mmm* SC to (A) heat at 53 °C and (B)
455 drying at 20 °C where \blacktriangle is adherent B103, \blacksquare is adherent Afadé, \square is planktonic Afadé and \triangle is
456 planktonic B103. An absence of a data point on the graph prior to the end of the experiment
457 indicates that no surviving cells were detected at that time point. Error bars represent standard
458 deviation.

459

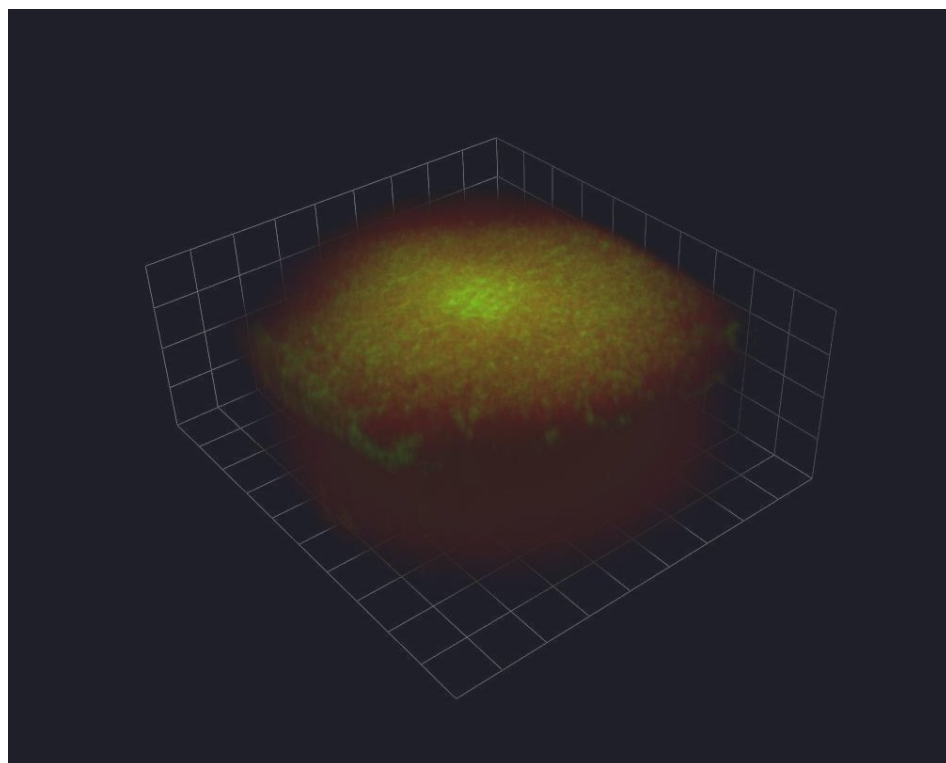
460 Figure 4. Effect of biofilm formation on resistance of *Mmm* SC to (A) oxidative stress of 100 mM
461 hydrogen peroxide and (B) 0.005 % SDS where \blacktriangle is adherent B103, \blacksquare is adherent Afadé, \square is
462 planktonic Afadé and \triangle is planktonic B103. An absence of a data point on the graph prior to the
463 end of the experiment indicates that no surviving cells were detected at that time point. Error
464 bars represent standard deviation.

465

466 Figure 5. 1D SDS PAGE of biofilm and planktonic forms of *Mmm* SC strains Afadé and B103
467 where lane 1; Afadé biofilm, lane 2, Afadé planktonic, lane 3, B103 biofilm, lane 4, B103
468 planktonic. Band A represents PTS system glucose-specific IIBC component, B is
469 phosphoenolpyruvate-protein phosphotransferase, C is elongation factor Tu, D is fructose-
470 bisphosphate aldolase class II and E is hypoxanthine phosphoribosyltransferase.

Figure 1.

A



B

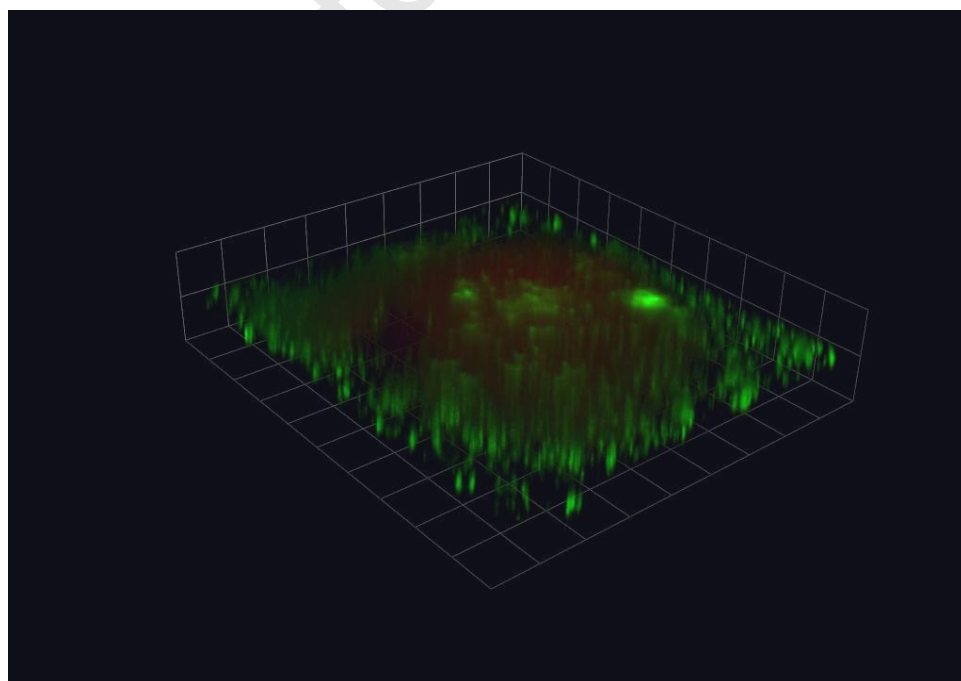
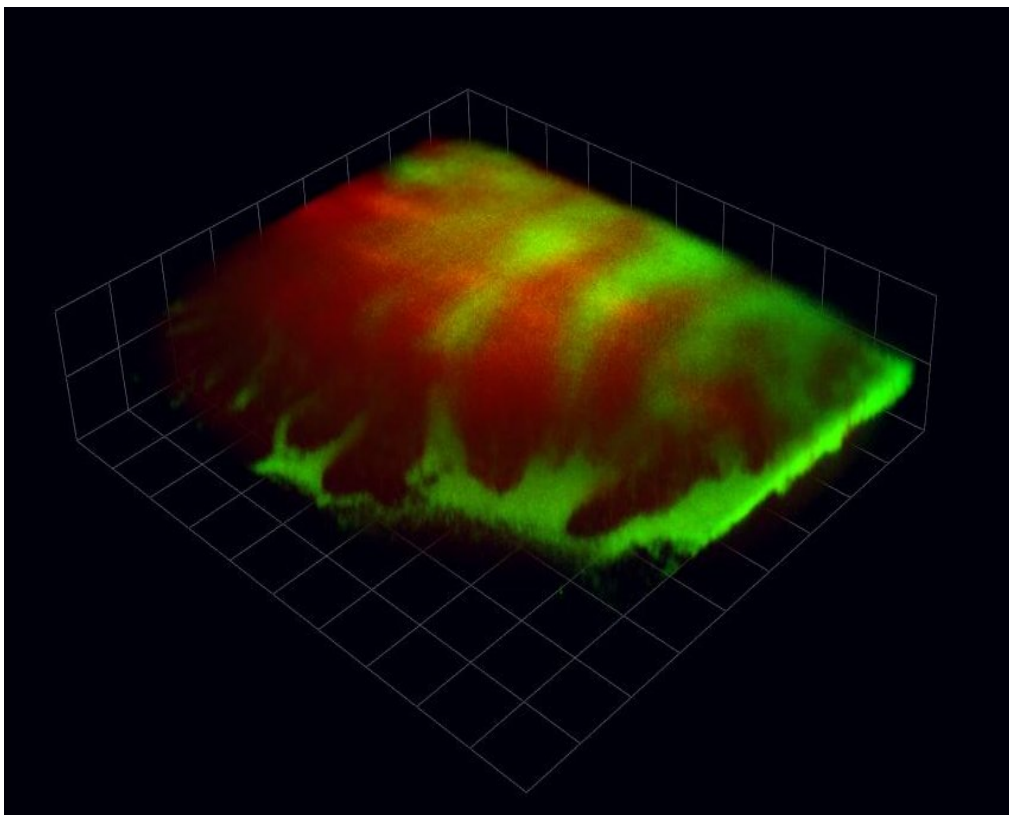


Figure 2.

A



B

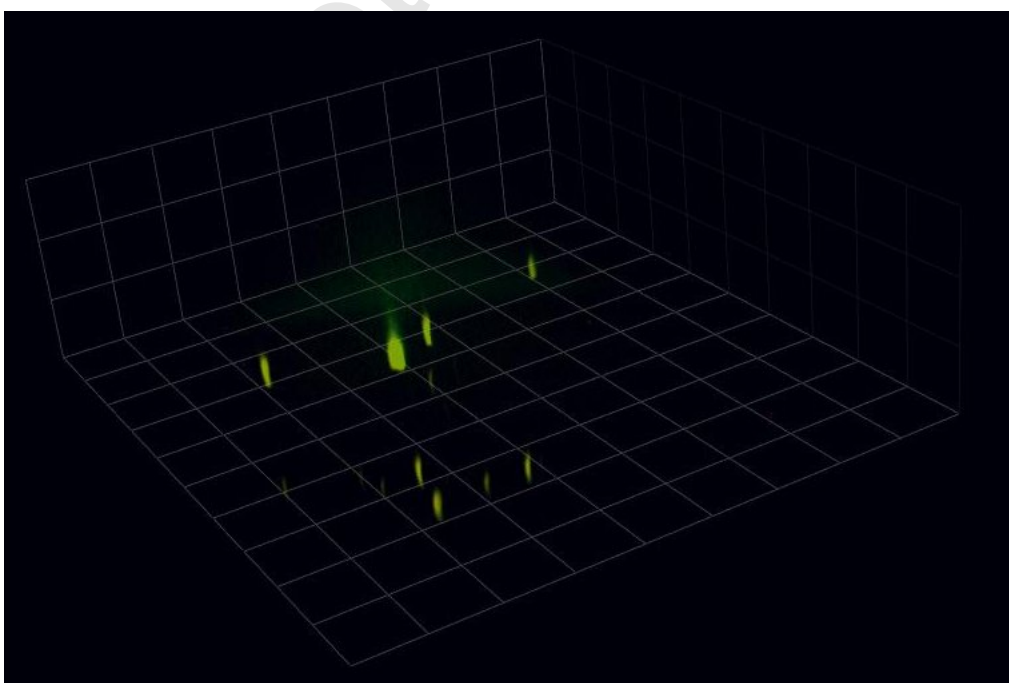


Figure 3.

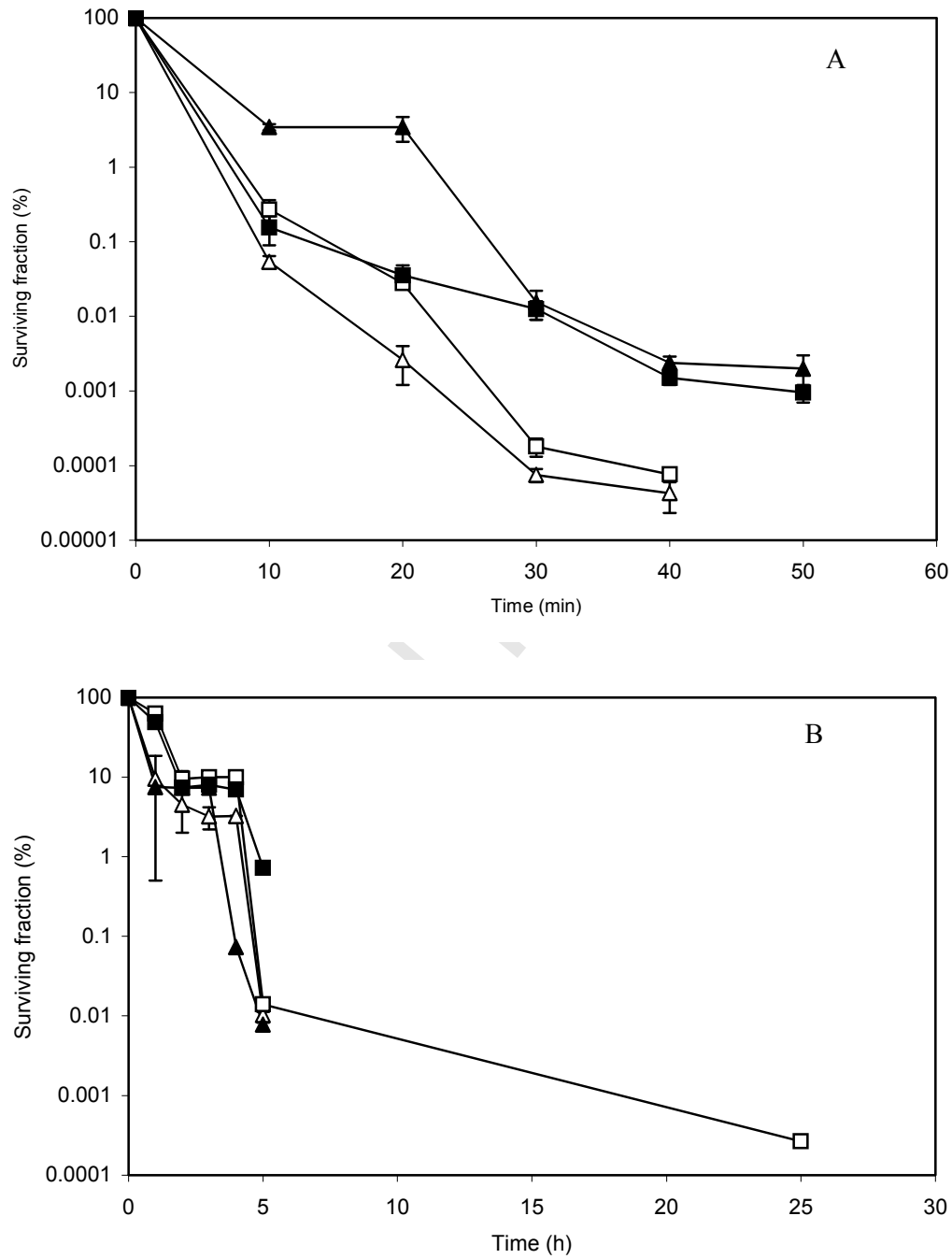


Figure 4.

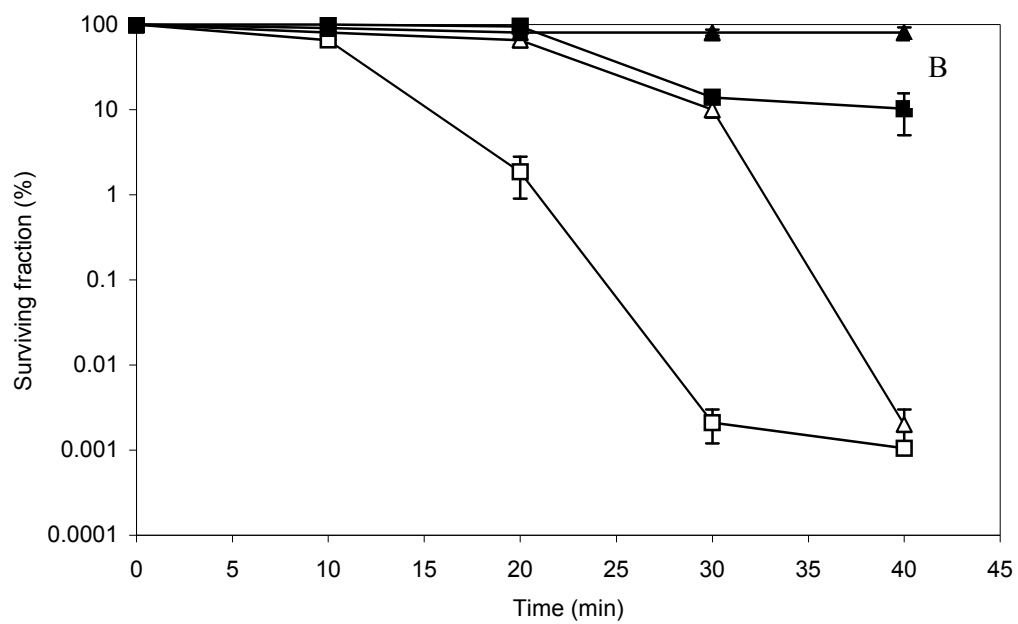
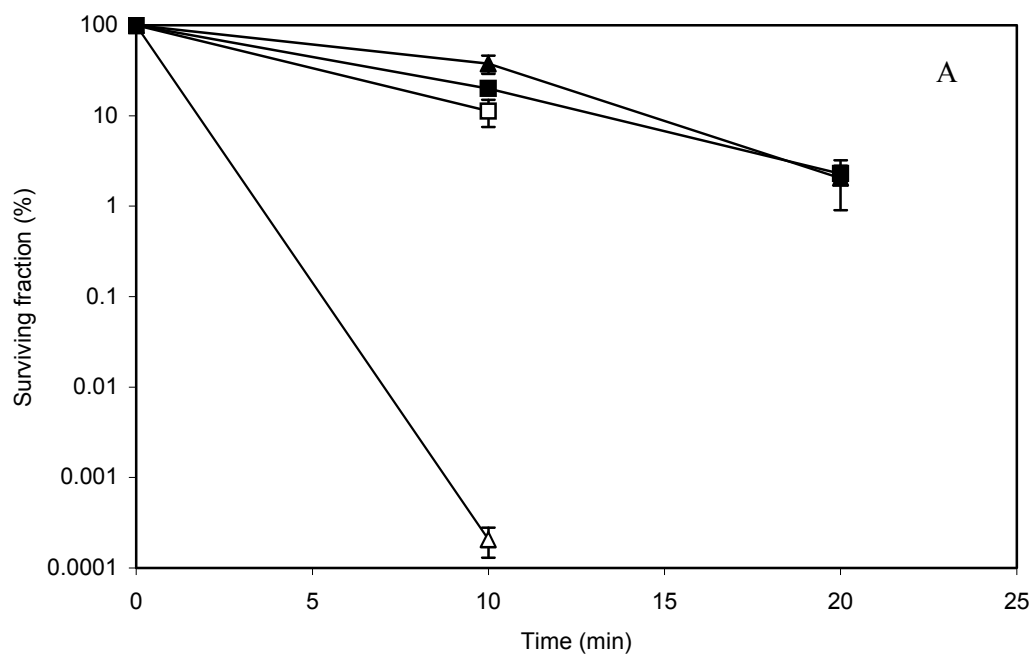


Figure 5.

