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

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Abstract

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

Introduction

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

Mycoplasmas possess small genomes and are thought to have evolved from more complex bacteria by reductive evolution losing many genes in the process. They lack many genes including those for cell wall synthesis, 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 in Razin, Yogev and Naot, 1998). Currently, very little is known about the pathogenesis of CBPP and of the persistence of *Mmm* SC in the host or the environment. Over a century ago, when the cause of the disease was unknown, following severe outbreaks in the USA there were indications that the organism may persist in the environment and it was written that " Many stables have been found in which the disease would appear and reappear after the

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

In many other bacterial species adherence to a solid surface and biofilm formation are important steps in the initiation of disease. Previously, we have demonstrated that some *Mycoplasma* species form biofilms and have speculated that this may enable them to persist in the environment and may even contribute to disease in the host (McAuliffe *et al.,* 2006). Mycoplasma biofilms were found to begin by a thin covering of individual cells followed by the formation of small groups of cells and after prolonged incubation larger groups of microcolonies that gave a characteristic stack-like structure with channels between stacks as seen in other bacterial biofilms were evident (McAuliffe et al, 2006). Although *Mycoplasma* species including *M. bovis*, *M. putrefaciens*, *M. cottewii* and *M. agalactiae* formed prolific biofilms and were more resistant to stresses including heat and desiccation when grown as a biofilm, we found that *Mmm* SC would not adhere to any of the surfaces we tested when an air/liquid interface model was used and cells were found to exist in a free-living state rather than adhere to a substratum (McAuliffe *et al*., 2006). However, in this study we will demonstrate that *Mmm* SC can be studied as an adherent layer when it is grown on a membrane placed on an agar plate in a simple biofilm model that lacks an air/liquid interface.

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

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

Materials and methods

Strains and growth conditions

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

Variation in biofilm formation among *Mmm* SC strains.

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

Heat shock and desiccation assay

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

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

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

Osmotic shock, oxidative stress and detergent stress assays

Stationary phase *Mmm* SC grown either as planktonic cultures in broth or as adherent cells on membranes on agar plates were subjected to stress of 5M NaCl or 100 mM H_20_2 or 0.005% sodium dodecylsulphate (SDS) for 50 min. Adherent cells were placed in a 30ml screw-topped tube (Sterilin) with 2 ml of stressor solution, while planktonic cells were added to a 30ml screw-topped tube (Sterlin) with 2 ml of the same solution to give a density of 10^6 cfu/ml. Triplicate samples were taken of both adherent and planktonic cells at 10 min to enumerate the number of surviving bacteria. Membranes or a 200 µl aliquot of the planktonic cells were aseptically removed from each treatment tubes and placed in a fresh tube containing volumes of with 1 ml and 800 µl of Eaton's broth respectively. Tubes containing membranes and planktonic cells were then vortexed for 1 min, and viable bacteria were enumerated as described above.

Long term survival assay

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

remaining determined by serial dilution as described above.

Confocal microscopy

Mmm SC biofilms were analysed by confocal laser microscopy. Biofilms were grown on membranes on Eaton's agar plates were stained using the BacLight bacterial viability assay kit according to the manufacturers instructions (Molecular Probes) live cells stain green with a SYT09 stain whereas dead cells with damaged membranes stain red with propridium iodide. Membranes were mounted onto glass slides and examined with a Perkin Elmer UltraView ERS spinning disk confocal microscope. For SYTO9, an excitation wavelength of 488 nm and an emission filter of 500-550 nm was used, while for propridium iodide, an excitation of 568 nm and an emission filter of 580-650 nm was used.

SDS PAGE and protein identification

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

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

Results

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

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

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

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

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

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

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

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

3. Changes in protein profile induced by adherence

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

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

Discussion

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

Bacterial biofilms are considered to be 10-1000 times more resistant than planktonic cells to all stress (Mah and O' Toole, 2001; Costerton, Stewart and Greenberg, 1999). Overall, adherent *Mmm* SC cells were significantly more resistant to the stresses they were exposed to during this study. Biofilm-grown *Mmm* SC were more resistant to peroxide and detergent stress, and heat stress than planktonic cells. These results are not surprising as for example, although not previously studied in Mycoplasma, resistance to hydrogen peroxide has been widely studied in other bacterial species where it has been shown that biofilms are considerably more resistant than planktonic cells (Elkins *et al*., 1999, Wen *et al*., 2005). Oxidative stress resistance is one of the key properties that enables pathogenic bacteria to survive the effects of reactive oxygen species produced by host cells (Storz and Zheng, 2000). Intriguingly, the production of hydrogen peroxide is also thought to be an important virulence factor for *Mmm* SC itself (Miles *et al*., 1991) but it remains unclear why the high levels of H_2O_2 that must be produced to act as a virulence factor are not toxic to the mycoplasmal cell. Mycoplasmas do not possess most of the enzymes that normally contribute to oxidative stress resistance in other bacterial species, such as catalase, and *Mmm* SC lacks most of the SoxR/SoxS oxidative shock regulon with the exception of the endonuclease IV *nfo* and the iron regulator *fur*. In addition, *Mmm* SC completely lacks the OxyR regulon that is crucial for oxidative stress resistance in many other bacterial species and even lacks a homolog to MsrA which protects against oxidative

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

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

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

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

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

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

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

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

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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

Figure legends

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

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

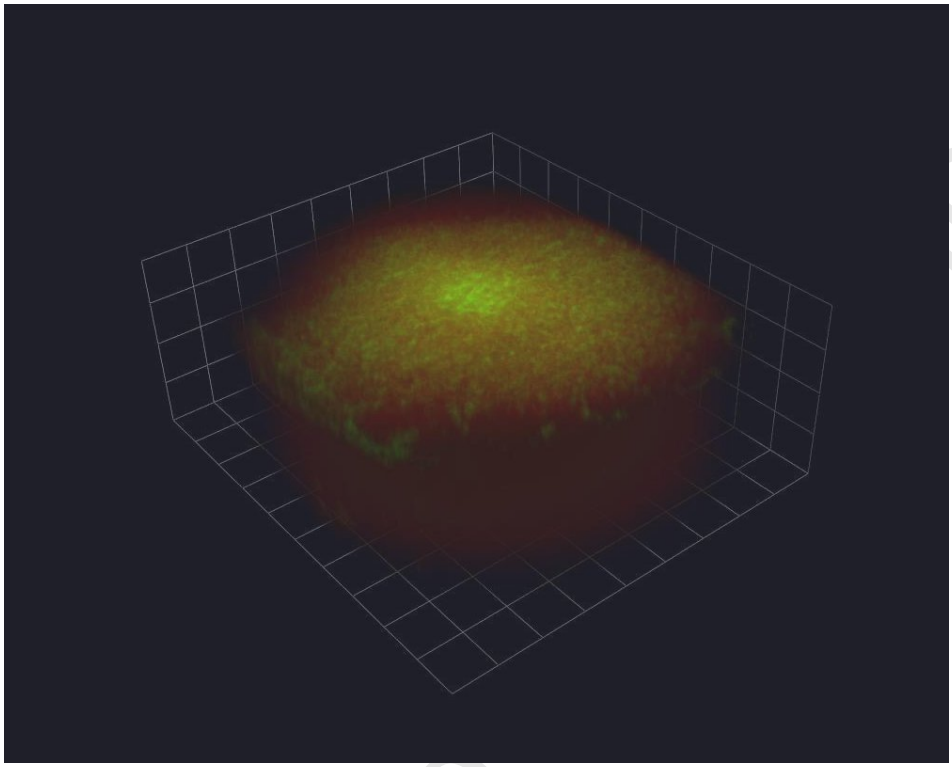
Figure 3. Effect of biofilm growth on the resistance of *Mmm* SC to (A) heat at 53 °C and (B) drying at 20 °C where ▲ is adherent B103, ■ is adherent Afadé, ☐ is planktonic Afadé and △ is planktonic B103. An absence of a data point on the graph prior to the end of the experiment indicates that no surviving cells were detected at that time point. Error bars represent standard deviation.

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

Figure 5. 1D SDS PAGE of biofilm and planktonic forms of *Mmm* SC strains Afadé and B103 where lane1; Afadé biofilm, lane 2, Afadé planktonic, lane 3, B103 biofilm, lane 4, B103 planktonic. Band A represents PTS system glucose-specific IIBC component, B is phosphoenolpyruvate-protein phosphotransferase, C is elongation factor Tu, D is fructose-bisphosphate aldolase class II and E is hypoxanthine phosphoribosyltransferase.

Figure 1.

A



B

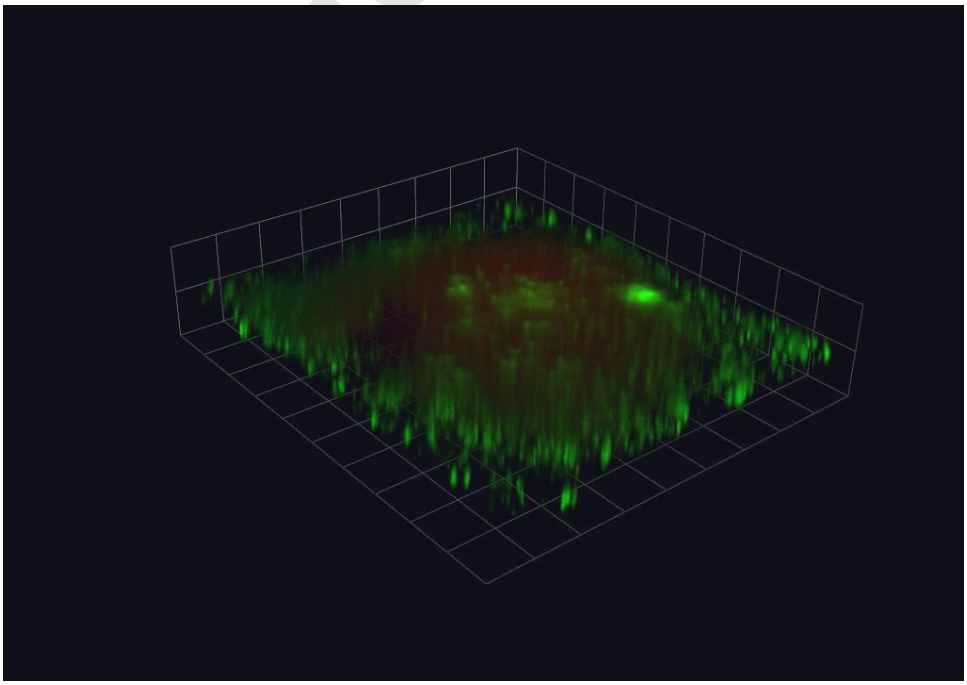
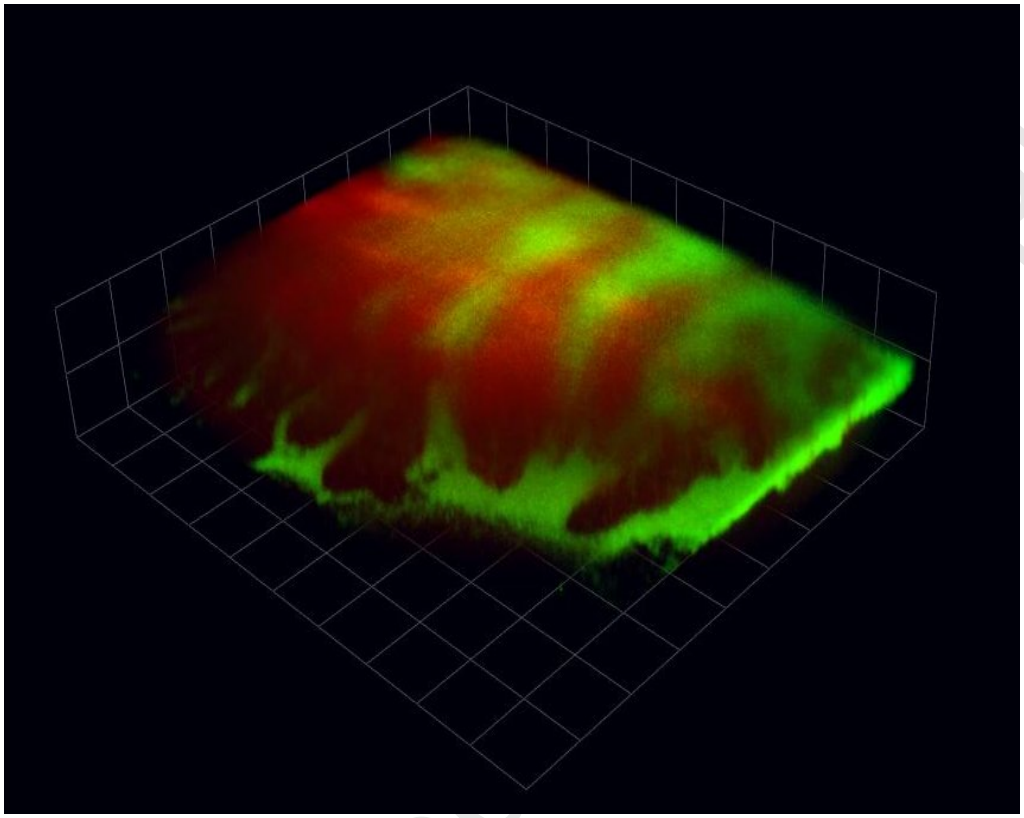


Figure 2.

A



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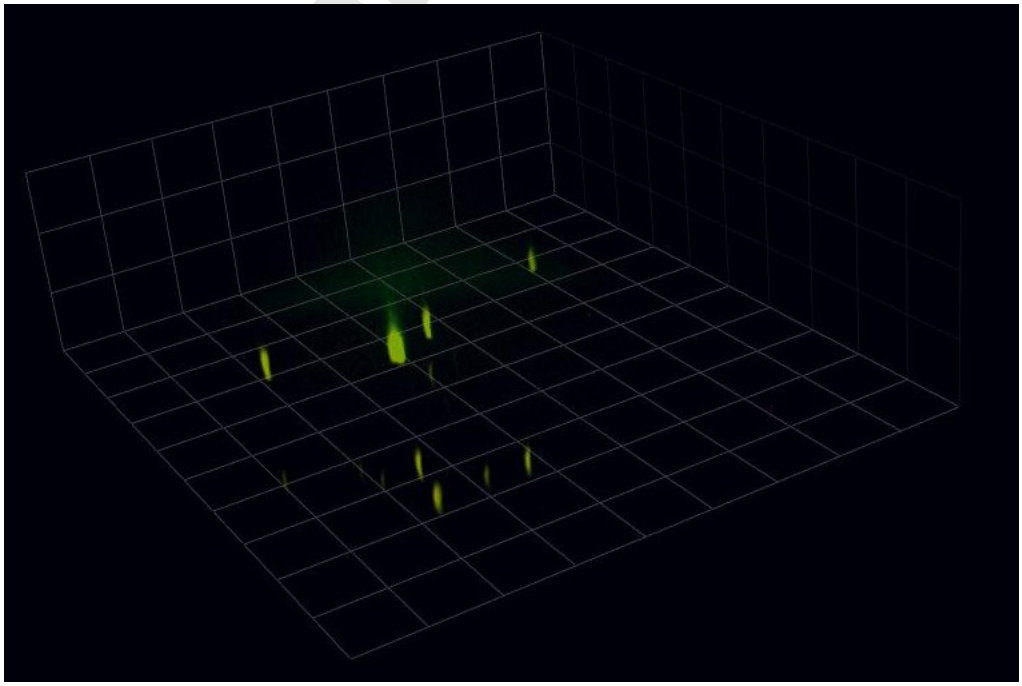


Figure 3.

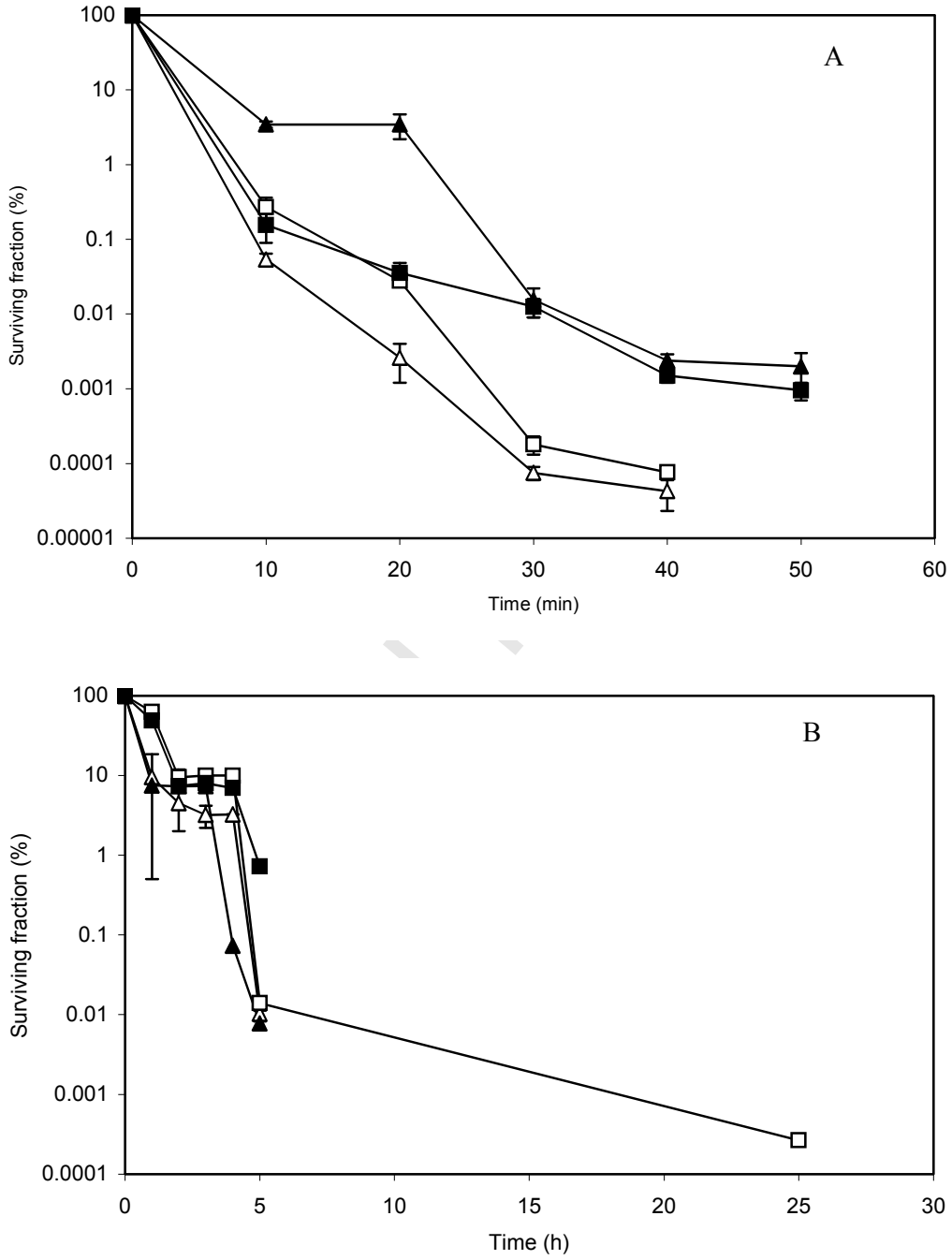
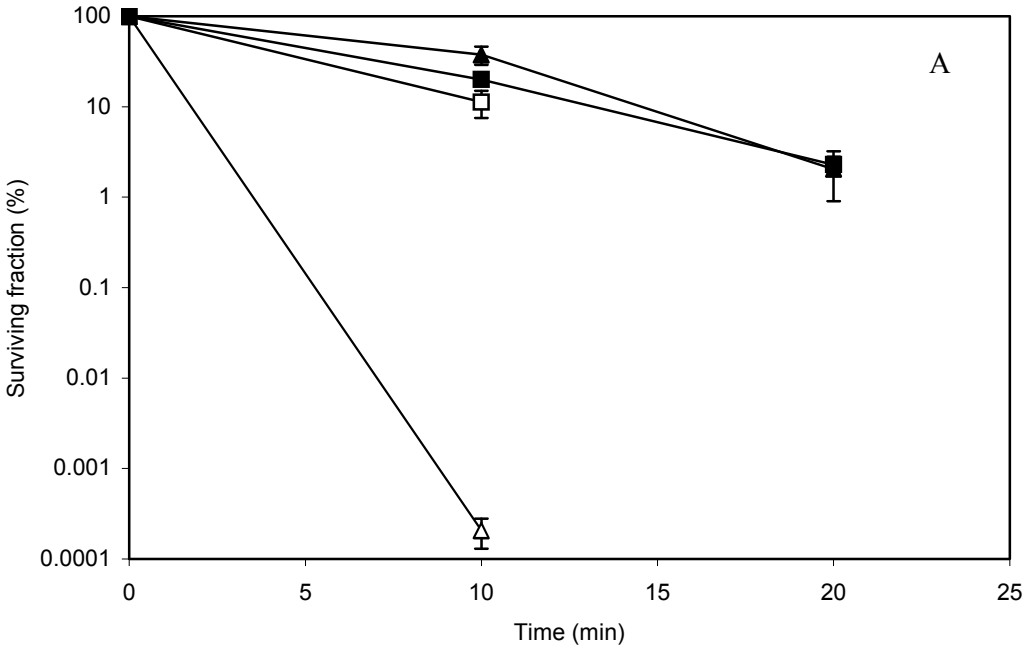


Figure 4.



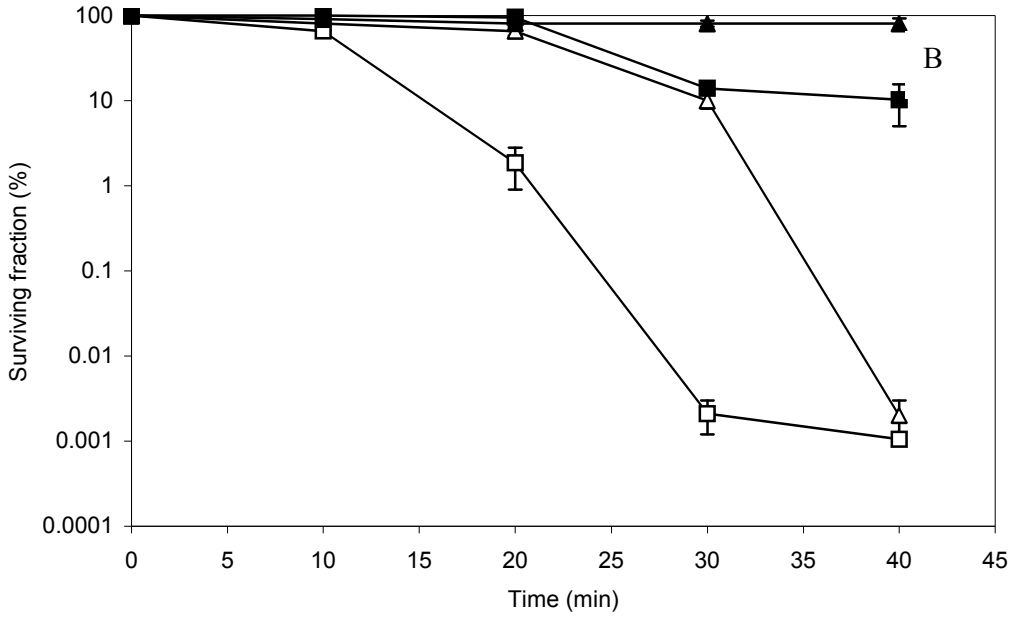


Figure 5.