RESEARCH LETTER

Comparison of *in vivo* and *in vitro* properties of capsulated and noncapsulated variants of *Mycoplasma mycoides* subsp. *mycoides* strain Afadé: a potential new insight into the biology of contagious bovine pleuropneumonia

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Abstract

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) strain Afadé had previously been shown to undergo spontaneous phase variations between an opaque capsulated variant and a translucent (TR) variant devoid of a capsule but able to secrete cell-free exopolysaccharides. This phase variation is associated with an ON/OFF genetic switch in a glucose permease gene. In this study, *in vivo* and *in vitro* assays were conducted to compare the virulence of the two variants and their abilities to resist host defence. Capsulated variants were shown, in a mouse model, to induce longer bacteraemia that was correlated with better serum resistance *in vitro*. In contrast, TR variants displayed better ability to adhere to an inert support, linked to the absence of a capsule, changes in cell surface hydrophobicity and increased resistance to antimicrobial peptide and hydrogen peroxide. The switch from one variant population to another, which was observed both *in vivo* and *in vitro* under stress conditions, is further discussed as a means for *Mmm* to modulate its interactions with animal hosts during different stages of the disease.

Introduction

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) is the causative agent of contagious bovine pleuropneumonia (CBPP), a severe disease of cattle notifiable to the world organisation for animal health. CBPP is currently widespread in sub-Saharan Africa where it threatens livestock production and limits trade exchange of animals, resulting in huge economic losses. CBPP remains a constant menace to European countries as shown by re-emerging outbreaks at the end of the last century (Nicholas *et al.*, 2009). CBPP is expressed as anorexia, fever and respiratory signs such as dyspnoea, polypnoea and nasal discharge. Animals that recover after the acute clinical phase may develop lung sequestra in which mycoplasmas are able to survive, resulting in chronicity of the disease (Provost *et al.*, 1987).

Although the mechanisms underlying the ability of *Mmm* to cause disease have been investigated for several years, few have been ascertained experimentally. The cytotoxicity of *Mmm* to bovine epithelial cells has been shown to rely on the capacity of mycoplasma to adhere to the host cells and generate hydrogen peroxide (Bischof *et al.*, 2008). The extracellular polysaccharide produced by *Mmm*, the so-called galactan, has also been associated with different degrees of clinical signs severity (Buttery *et al.*, 1976; March *et al.*, 1999). This galactan, a polymer of β(1→6) linked galactofuranose residues, has been shown to be either capsular (CPS; Plackett & Buttery, 1963) or cell-unbound [exopolysaccharide (EPS)] (Bertin *et al.*, 2013). *Mmm* strains that produce higher amounts of CPS were found to induce longer septicaemia in a mouse model (March & Brodlie, 2000). Although large quantities of EPS were detected in the body fluids of *Mmm*-infected animals during the acute phase of the disease (Turner, 1962), the exact role of EPS secretion in the pathological process has yet to be elucidated.

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Experiments showed that intravenous injection of EPS resulted in hyperpnoea followed by coughing and salivation in cattle (Hudson *et al.*, 1967; Buttery *et al.*, 1976). Recently, we showed that *Mmm* PG1^{TS} yielded two colony variants on solid medium, that is opaque (OP) and translucent (TR) variants, that either produce a capsule (OP) or secrete EPS (TR) (Bertin *et al.*, 2013). In *Mmm* strain Afadé, this phenotypic variation has been associated with a genetic ON/OFF switch in a glucose permease gene that results in premature termination of the protein PtsG and hence loss of an epitope (Gaurivaud *et al.*, 2004). The characteristics of the two variants are summarised in Table 1.

In the absence of a cell wall, phase variation has long been considered as a way for mycoplasmas to rapidly alter their surface antigens and hence escape the host immune response or more generally modulate their interactions with the host (Citti *et al.*, 2010). The *Mmm* model is interesting as the antigenic variation of PtsG is further associated with the presence/absence of a capsule (Bertin *et al.*, 2013). In other bacterial models, such as *Streptococcus pneumoniae*, variation between capsulated or noncapsulated variants was shown to be associated with different levels of virulence (Hava *et al.*, 2003). In *Mmm*, spontaneous phase variation between OP and TR variants had been observed in the absence of host-related selection pressure *in vitro* (Gaurivaud *et al.*, 2004), suggesting that it might be stochastically modulated. This contrasts with observations in other bacterial models, such as *Klebsiella pneumoniae*, in which loss of the capsule was considered as an evolutionary dead end (Struve & Krogfelt, 2003). This study was conducted to determine whether switching from OP to TR variants or vice versa could play a part in the different phases of disease development. We first estimated the virulence of each variant in a mouse model that had previously been validated by comparing the bacteraemia induced by intraperitoneal inoculation of attenuated vs. freshly isolated *Mmm* strains, in the absence of any clinical signs (Smith, 1968). We then explored, *in vitro*, several growth or host immune defence resistance mechanisms that might distinguish the two variants and hence help decipher the biological significance of surface variations.

Table 1. Properties of TR and OP variants isolated from *Mmm* strain Afadé

	TR variants	OP variants
Colony opacity	Translucent	Opaque
Galactan capsule	No	Yes
Galactan cell-free exopolysaccharide	+++	+/–
Glucose PTS permease (MSC_0860)	Wild-type	Truncated
3F3 monoclonal detection*	Yes	No
Reversion frequencies in PPLO broth (per cell per generation)	From TR to OP 10^{-4} to < 10^{-6}	From OP to TR 10^{-2} to < 10^{-6}

OP, opaque; TR, translucent.

*Monoclonal antibody 3F3 targets the glucose permease MSC_0860 (*Mmm* PG1^{TS} locus tag) and was shown to bind TR, but not OP variants (Bertin *et al.*, 2013).

Materials and methods

Mycoplasma variants, growth conditions and characterisation

OP and TR variants of the *Mmm* strain Afadé (Bertin *et al.*, 2013) were grown at 37 °C under 5% CO_2 in PPLO-based medium supplemented as described previously (Poumarat *et al.*, 1991). The effect of pH on TR variants viability was investigated using PPLO broth buffered with 50 mM HEPES pH 7.5. A chemically defined medium CMRL-1066, previously shown to sustain viability but not mycoplasmal growth (Bertin *et al.*, 2013), was used for hydrogen peroxide, gramicidin, adhesion and serum survival assays. Viable cell concentration (CFU mL^{-1}) was determined by preparing appropriate serial dilutions of broth cultures and plating on PPLO agar plates. The proportion of TR and OP variants was monitored by colony blotting with 3F3 monoclonal antibody (Gaurivaud *et al.*, 2004).

Experimental infection of mice

Two groups of 26 females C57BL/6JIco mice received an intraperitoneal inoculation of 0.5 mL of PPLO broth containing 2×10^8 CFU mL^{-1} of either the TR or OP variant. Individual blood samples (*c.* 100 μL) were retrieved 2–12 days after inoculation and serially diluted in PPLO broth. After incubation for 7 days at 37 °C, *Mmm* was detected by MF-Dot test using either the SC11 polyclonal antibody or 3F3 monoclonal antibody (Brocchi *et al.*, 1993) and by nested-PCR at 5 d.p.i. (days post inoculation) (Miserez *et al.*, 1997). The results were statistically analysed by chi-square test. The animals were housed in our approved experimental facilities (no. A 69 387 0801) and cared for under the supervision of a licensed professional (LL: 96 387 191), in accordance with the EC Directive 86/609/EEC.

In vitro metabolic activity

The metabolic activity of stationary phase cultures of the OP and TR variants (10^8 CFU mL^{-1}) was assessed by XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] reduction (Babu *et al.*, 2012) using the TOX2 kit (Sigma-Aldrich, Saint Quentin Fallavier, France). Two hundred microlitres of mycoplasmas

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were incubated with 40 μL XTT (1 mg mL^{-1}) for 4 h at 37 °C, and absorbance was measured at 450 nm against a negative control composed of noninoculated PPLO broth incubated with XTT.

Serum bactericidal and gramicidin killing assays

Mycoplasma cells grown to stationary phase in PPLO broth were washed and resuspended in CMRL-1066 at 10^6 CFU mL^{-1}. One hundred microlitre aliquots were mixed with an equal volume of 20% guinea pig serum (GPS) or heat-inactivated GPS (56 °C, 1 h). After 90 min at 37 °C, the cells were counted and the percentage of surviving mycoplasmas was calculated as the fraction of CFU recovered after treatment with GPS in relation to that recovered after treatment with heat-inactivated GPS. For the gramicidin killing assays, 1 mL of 10^6 CFU mL^{-1} was incubated for 2 h at 37 °C with 1 μg mL^{-1} of gramicidin (Sigma-Aldrich) or PBS only, as a negative control. The percentage of surviving mycoplasmas was expressed as the fraction of CFU after gramicidin treatment in relation to that obtained after incubation with PBS only.

Hydrogen peroxide sensitivity assays

Stationary phase PPLO cultures were diluted in CMRL-1066 (10^5 CFU mL^{-1}). Cell counts and the proportion of variants were determined before and after incubation for 15 and 30 min at 37 °C with 10 mM H_2O_2. The survival percentage was determined from the fraction of CFU recovered before H_2O_2 addition in relation to the CFU recovered after incubation with H_2O_2.

Adherence test

TR and OP variants cultures (48 h, 10^7 CFU mL^{-1}) were diluted 1000-fold in PPLO broth or in a mix of CMRL-1066 and PPLO broth at 1 : 1 dilution. Polystyrene microtitre plates were inoculated with the dilutions, incubated at 37 °C for 4 days, washed with 1× PBS and dried for 45 min at 55 °C. Adherent cells were stained with 0.5% crystal violet in 1× PBS (30 min, room temperature). The wells were washed five times with 1× PBS and left to dry at room temperature. The crystal violet was then dissolved with 30% acetic acid and 70% ethanol, and the $OD_{580\ nm}$ was recorded. Noninoculated broth was used as negative control.

SDS-PAGE and protein identification

SDS-PAGE was performed as described previously (Gaurivaud *et al.*, 2004). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for protein identification was performed by the proteomics platform of the French National Institute for Agricultural Research (http://pappso.inra.fr/) following a standard protocol. Protein identification was performed against the protein database of *Mmm* strain PG1 (NC_005364.2).

Cell surface hydrophobicity test

Cell hydrophobicity was measured using the ammonium sulphate test (Lindahl *et al.*, 1981). Four millilitres of mycoplasma cultures were centrifuged for 20 min (10 000 ***g***) and resuspended in 200 μL of 1× PBS. Twenty microlitres of bacterial suspension were mixed with 200 μL of ammonium sulphate solutions (0–4.0 M). Aggregation was recorded after 1 h of mixing at room temperature on a shaker.

Results

OP variants induced longer mycoplasmaemia in a mouse model

The virulence of TR and OP variants *in vivo* was investigated by intraperitoneal injection of two experimental groups of 26 mice with 10^8 CFU of the TR or OP variants, respectively. The resulting mycoplasmaemia was then monitored by blood culture and MF-dot. The OP variants induced significantly longer mycoplasmaemia than the TR variants (Table 2). At 5 d.p.i., mycoplasma could no longer be detected in blood from mice inoculated with the TR variants. In contrast, in mice inoculated with the OP variants, mycoplasmaemia was observed in 2/26 animals at 5 and 7 d.p.i. At 5 d.p.i., the difference in mycoplasmaemia was further confirmed by a PCR assay known to me more sensitive than MF-dot (10/26 vs. 0/26 positive cultures for the OP and TR variants, respectively). Furthermore at 7 d.p.i., the originally injected OP variants were shown to revert to TR variants in one of the two mycoplasma-positive mice (Table 2).

The longer mycoplasmaemia observed with the OP variants could result from (1) failure of the host response to eliminate the mycoplasmas and/or (2) enhanced multiplication of the bacteria within the host. Both hypotheses were further explored *in vitro*.

OP variants displayed a longer stationary growth phase *in vitro*

Growth and survival of TR and OP variants over an extended culture period were compared *in vitro* in a broth medium containing heat-inactivated sera only (Fig. 1). Both variants displayed similar exponential and

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Table 2. Duration of mycoplasmaemia following infection of mice

Days postinoculation (d.p.i.)	Number of mice displaying mycoplasmaemia after infection*	
	TR variants (n = 26)	OP variants (n = 26)
2	22	23
3	17	17
4	8	9
5	0†	2†
6	0	3
7	0	2‡
12	0	0

OP, opaque; TR, translucent.

**Mmm* was detected by culture and identified by MF-dot as described in Materials and methods.

†At 5 d.p.i., the numbers of septicaemic mice in the OP and TR-inoculated groups were significantly different with a *P*-value of 0.019 using the chi-square test.

‡TR-variant revertants were detected in the blood culture of one mouse.

early stationary phases of growth up to 72 h. A longer incubation period resulted in a steady drop in mycoplasmal titres in both cultures but with different dynamics, the decay of viable cells being slower for OP than for TR variants for which no cell survived after 248 h at 37 °C (Fig. 1). As a consequence of the metabolic activity, the pH of the culture medium decreased from 7.7 to 6.4 for OP, after 224 h of incubation, but fell to 5.4 for the TR variants (Fig. 1). Hence, in the absence of host interaction, the longer survival of OP variants might be due to their propensity to only moderately acidify their growth medium. This was further confirmed by the observation of a longer stationary growth phase when TR variants were grown in PPLO broth buffered with 50 mM HEPES (Fig. 1). Under these conditions, the metabolic activity of TR variants, estimated from the reduction of an XTT dye, was comparable to that of the nonbuffered culture of OP variants, after 56, 200 and 224 h of incubation (Fig. 1). These data suggest that both variants survive in a similar metabolically active state when the pH remains stable, which is the case in blood due to pH homoeostasis. All together, our results suggest that the difference in mycoplasmaemia observed *in vivo* might result from different host interactions rather than from intrinsic growth characteristics of the variants.

OP and TR variants showed different resistance patterns to the host immune response

We first investigated whether the better persistence of OP variants in our mouse model was related to a better capacity to survive the bactericidal activity of serum. After incubation of the TR and OP variants with GPS *in vitro* for 90 min at 37 °C, 90% of the OP variants were recovered compared with < 50% of the TR variants (Fig. 2). This result demonstrated that OP variants were more resistant to the lytic action of GPS, which could in turn explain their longer persistence in mice.

In the infected host, *Mmm* is also exposed to the potent antimicrobial arsenal deployed by the phagocytes. This aspect of host interaction was approached by testing the bactericidal activity of antibiotic peptides and oxidative molecules against the two variants. The TR variants were found to be more resistant to exposure to 10 mM hydrogen peroxide and 1 μg mL^{-1} gramicidin than the OP variants (Fig. 2). The lethal concentration of gramicidin for OP variants was 2 μg mL^{-1}, whereas TR variants were detected up to a gramicidin concentration of 8 μg mL^{-1} (data not shown). Hence, in our mice model, the longer survival of OP variants was not due to a better resistance to oxidative stress or antimicrobial peptides. Interestingly, TR revertants were detected *in vivo* at 7 d.p.i. in one mouse inoculated with OP variants (Table 2). This suggests that the different resistance patterns observed *in vitro* could provide a means of adapting to the host defence *in vivo* by switching from one variant to another.

The ratio of OP and TR variants varied under stress conditions

To investigate whether an oxidative stress could modify the composition of the *Mmm* strain Afadé population, 10 mM hydrogen peroxide was applied to an OP variants population which contained only 0.5–0.7% TR variants (Fig. 3a). After 30 min at 37 °C, the percentage of TR variants in the OP population had risen to 15–22% (Fig. 3b). This finding indicated that the proportion of OP/TR in *Mmm* strain Afadé could rapidly evolve in response to an oxidative stress during the course of the infection.

Very surprisingly, we also found that prolonged incubation of the OP population *in vitro* gave rise to a subpopulation mainly composed of TR variants. After 224 h, the OP population declined dramatically (Figs 1 and 3c), and this was attributed to the depletion of nutrients as the stationary-phase OP variants were metabolically active. Surprisingly, although no OP variants were able to grow after 272 h, some TR variants were detected. A second growth curve, composed exclusively of TR variants, was observed up to 512 h of incubation at 37 °C (Fig. 3c). These data indicate that a reversion from OP to TR variants allowed an increased survival of *Mmm* strain Afadé under conditions of nutrient deficiency. Long-term survival of the Afadé strain, up to 20 weeks at 20 °C, had previously been observed *in vitro* and had been linked to

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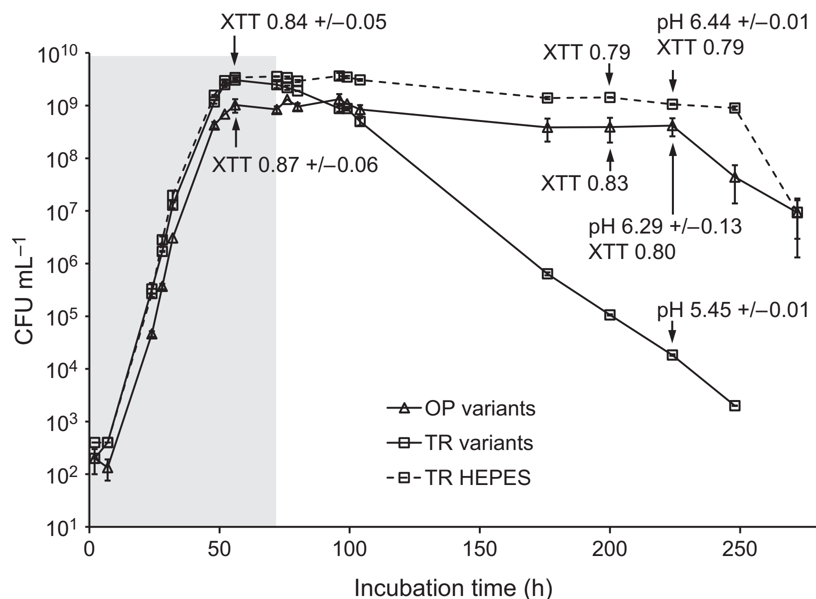

Fig. 1. Growth and persistence of TR and OP variants in PPLO broth. Data are the average CFU mL^{-1} obtained from two different experiments with TR variants and TR cultivated in PPLO-buffered medium (TR HEPES) and from three experiments with OP variants. Error bars indicate standard deviations. PPLO broth acidification was measured after incubation for 224 h. XTT values for 10^8 CFU after incubation for 56, 200 and 224 h are indicated. Grey shading indicates exponential and early stationary phases of growth.

enclosure of the mycoplasmal cells in a biofilm structure (McAuliffe *et al.*, 2008). We therefore investigated whether the OP and TR variants differed in their capacity to form a biofilm.

Surface properties of the variants impacted their attachment to an inert support

As bacterial adhesion to surfaces is the initial step of biofilm formation (Flemming & Wingender, 2010), we investigated the ability of our variants to adhere to polystyrene plates. The TR variants exhibited better adhesion to this inert surface, which was further enhanced when assayed in a mix of PPLO and CMRL-1066 (1 : 1 v/v) (Fig. 4). Under these latter conditions, the surface adhesion of TR variants might have been increased due to nutrient limitation, as observed for other bacteria (Petrova & Sauer, 2012). The different adhesion ability of the two variants might be related to their different surface properties, one being capsulated, the other not. Using the agglutination test (Lindahl *et al.*, 1981), we confirmed that TR variants were precipitated by 2.0 M ammonium sulphate, as compared with 3.5 M for the OP variants. The increased hydrophobicity of the cell surface of TR variants, expected in the absence of CPS (Marshall *et al.*, 1995), could also explain their better adherence to a hydrophobic surface such as polystyrene. Preliminary observations of the proteome of each variant by SDS-PAGE revealed

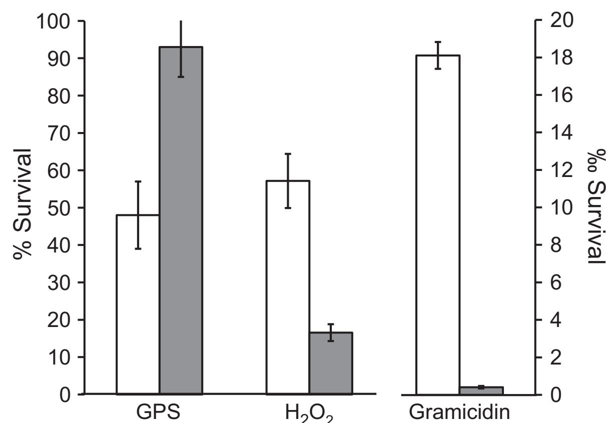

Fig. 2. Effect of hydrogen peroxide, GPS and gramicidin on the survival of TR (white) and OP (grey) variants. Mycoplasmas were incubated with 10 mM hydrogen peroxide for 15 min at 37 °C, 20% GPS for 90 min at 37 °C or 1 μg mL^{-1} gramicidin for 120 min at 37 °C. Data are the averages of three biological repeats. Error bars indicate standard deviations.

similar banding patterns. However, two high molecular weight proteins (201 and 206 kDa) were missing from the TR variants (Supporting Information, Fig. S1). These proteins were identified by LC-MS/MS as MSC_0033 and MSC_0457 (*Mmm* strain $PG1^{TS}$ locus tag), for which the *in silico* predicted membrane-location had been recently

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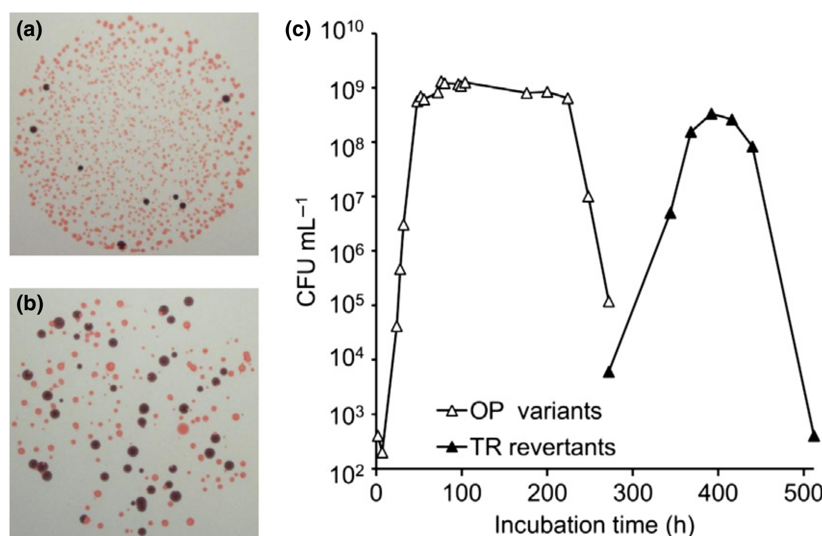

Fig. 3. Short- and long-term selection of opacity variants under stress conditions. (a) Before and (b) after 10 mM hydrogen peroxide treatment for 30 min at 37 °C. TR and OP variants colonies are dark blue and pink coloured, respectively. (c) OP variants were cultivated for 3 weeks in PPLO broth. Variants were discriminated by colony blotting with 3F3 monoclonal antibody.

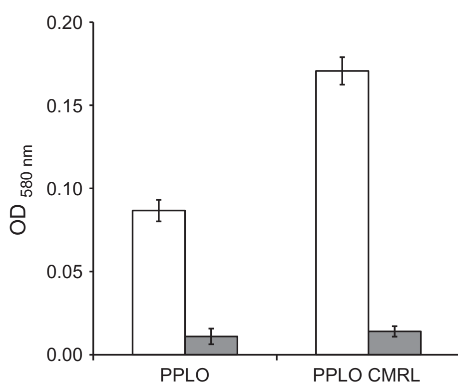

Fig. 4. Adhesion of TR (white) and OP (grey) variants on polystyrene plates. TR and OP variants were incubated in PPLO broth or PPLO CMRL (1 : 1 dilution) at 37 °C for 4 days. Data are the average absorbances obtained from six different experiments. Error bars indicate standard deviations.

confirmed by analysing the surface proteome of strain Afadé (Krasteva *et al.*, 2014). Our results suggest that the surfaces of OP and TR cells differed not only in the presence of a capsule but also in the differential expression of membrane proteins, which could modulate their overall surface hydrophobicity and their attachment to an inert support.

Discussion

To be a successful pathogen, a bacterium must infect its host, multiply despite the immune response, colonise deeper organs and finally result in cellular, tissue or organ damage (Casadevall & Pirofski, 2000). In view of the usual clinical outcome of CBPP (Provost *et al.*, 1987), there can be no doubt that *Mmm* is a successful pathogen. However, during the course of the disease, from infection to cellular damage, the interplay between *Mmm* and its host is clearly multiphasic. The *Mmm* strain Afadé is known to be highly pathogenic (Abdo *et al.*, 1998) and our recent findings that it undergoes intrastrain phase variation *in vitro*, associated with differential capsule-production capacities (Bertin *et al.*, 2013), provide an interesting model to explore the host-pathogen interplay. In the present study, the virulence of the OP and TR variants was characterised using a mouse model previously demonstrated to accurately mirror virulence findings in a bovine host (Smith, 1968) and by *in vitro* experiments aimed at deciphering those properties of the variants that might modulate their host-interaction during the course of infection.

We showed *in vivo* that the OP capsulated variants survived significantly longer than TR variants, in the bloodstream of infected mice. We hypothesised that the capsular material enhanced their resistance to innate immune responses such as phagocytosis and serum killing, both being first-line mechanisms for bacterial clearance (Miajlovic & Smith, 2014). Indeed, *in vitro*, we demonstrated that the OP variants were more resistant to serum killing and were able to actively multiply with limited acidification of their environment. These findings suggest that the OP variants might be adapted for active but discreet multiplication in the bloodstream of infected animals and thus for dissemination.

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In contrast, we propose that the TR variants might be better adapted to certain host tissues or microniches. Firstly, the TR variants did not resist serum killing and showed a better capacity to adhere to abiotic surfaces. This latter property is very likely related to the absence of a capsule as similar observations were reported for *S. pneumoniae*, in which the loss of CPS resulted in increased surface attachment and consequently biofilm formation (Moscoso *et al.*, 2006). Furthermore, the TR variants secrete EPS, which are considered major constituents of bacterial biofilms (Flemming & Wingender, 2010) and possess a functional *ptsG* gene (Gaurivaud *et al.*, 2004; Bertin *et al.*, 2013) that has previously been demonstrated to display upregulated expression in Afadé biofilm (McAuliffe *et al.*, 2008). On the basis of these properties, the TR variants were hypothesised to be more efficient at tissue, notably lung, colonisation, as also observed for TR variants of *S. pneumoniae* (Kim & Weiser, 1998). This implies that TR variants have to resist lung defences such as antimicrobial peptides and H_2O_2 secreted by the phagocytes, the first line of defence against mycoplasmas invading the lung (Sibille & Reynolds, 1990). Our *in vitro* results clearly indicated that TR variants were better adapted to resist active oxygen species and antimicrobial peptides such as gramicidin, than OP variants. Resistance to H_2O_2 could be mediated by the EPS secreted by the TR variants, as suggested in earlier studies (Duan & Kasper, 2011). With respect to gramicidin resistance, the overall cell surface hydrophobicity of the TR variants could be involved.

In conclusion, the OP and TR variants of *Mmm* strain Afadé exhibited different and complementary properties *in vitro* that could partly explain the different levels of mycoplasmaemia induced in a mouse model. Further investigations have yet to be conducted to fully elucidate the advantages of each variant in their natural hosts. However, such investigations are likely to be limited by the difficulty of reproducing CBPP experimentally in bovine (March & Brodlie, 2000). We demonstrated that, *in vitro*, the genetic switch from one variant to another led to the rapid selection of a variant subpopulation adapted to its microenvironment. This could be a real asset *in vivo*, enabling *Mmm* to modulate its degree of virulence during the different stages of the disease and also during the final stages, that is transmission to a new host or transition towards chronicity.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Polyacrylamide gel electrophoresis of whole cell extracts from TR and OP variants.

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