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Galactofuranose in *Mycoplasma mycoides* is important for membrane integrity and conceals adhesins but does not contribute to serum resistance

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Summary

***Mycoplasma mycoides* subsp. *capri* (*Mmc*) and subsp. *mycoides* (*Mmm*) are important ruminant pathogens worldwide causing diseases such as pleuropneumonia, mastitis and septicaemia. They express galactofuranose residues on their surface, but their role in pathogenesis has not yet been determined. The *M. mycoides* genomes contain up to several copies of the *glf* gene, which encodes an enzyme catalysing the last step in the synthesis of galactofuranose. We generated a deletion of the *glf* gene in a strain of *Mmc* using genome transplantation and tandem repeat endonuclease coupled cleavage (TREC) with yeast as an intermediary host for the genome editing. As expected, the resulting YCp1.1-Δ*glf* strain did not produce the galactofuranose-containing glycans as shown by immunoblots and immuno-electronmicroscopy employing a galactofuranose specific monoclonal antibody. The mutant lacking galactofuranose exhibited a decreased growth rate and a significantly enhanced adhesion to small ruminant cells. The mutant was also 'leaking' as revealed by a β-galactosidase-based assay employing a membrane impermeable substrate. These findings indicate that galactofuranose-containing polysaccharides conceal adhesins and are important for membrane integrity. Unexpectedly, the mutant strain showed increased serum resistance.**

Introduction

Infectious diseases caused by members of the genus *Mycoplasma* feature prominently on the list of bacterial pneumonias in humans and in diverse livestock species. Currently, disease control depends to a large extent on the use of antimicrobials for treating human mycoplasma infections, and on suboptimal vaccines and movement restriction combined with 'test and slaughter' policies for controlling ruminant diseases. The use of antimicrobials bears the risk of generating resistant *Mycoplasma* spp., as recently observed in China (Zhao *et al.*, 2013) as well as promoting the development of other resistant bacteria. Various empirical approaches have been followed to develop vaccines for infectious diseases caused by

Mycoplasma spp., but the current lack of effective vaccines shows the limitations of such approaches that can potentially be overcome by rationally designed novel vaccines (Rueckert and Guzman, 2012; Jores *et al*., 2013).

Contagious bovine pleuropneumonia (CBPP) caused by *M. mycoides* subsp. *mycoides* (*Mmm*), a recently evolved pathogen (Dupuy *et al*., 2012; Fischer *et al*., 2012), is a major livestock disease in Africa that is currently spreading and affects most countries in sub-Saharan Africa. This spread can be attributed to factors such as financial constraints, poor veterinary services and the difficulties of disease control in traditional pastoralist systems and cannot be prevented by the current live vaccine that confers limited immunity, which lasts for only up to 1 year (Thiaucourt *et al*., 2000; Jores *et al*., 2013).

The development of an efficient vaccine has been hampered by a lack of understanding of host–pathogen interactions during disease. Until recently, the mutagenesis techniques available for *M. mycoides* such as transposon mutagenesis were inefficient, and the results were ambiguous. Transposon insertions can affect expression of neighbouring genes through homology-based silencing and/or read-through activity of regulatory elements (Rebollo *et al*., 2012). Moreover, transposons quite often have integration site preferences. The lack of suitable genetic tools to perform site-directed mutagenesis in many *Mycoplasma* species has hampered our current understanding of their biology and interactions with their hosts. However, with the recent advent of synthetic genomics, *M. mycoides* subsp. *capri* (*Mmc*), the closest relative of *Mmm*, has become a model organism for studies on whole genome synthesis (Gibson *et al*., 2010), genome transplantation (Lartigue *et al*., 2007) and genome editing using tandem repeat endonuclease coupled cleavage (TREC) (Noskov *et al*., 2010), which exploit yeast as an intermediary host for the mycoplasma genome (Lartigue *et al*., 2009). These new techniques now open up to a new era in mycoplasma research, where site-directed mutagenesis can be performed in a systematic and efficient manner to understand the biology and pathogenesis of the bacteria.

Carbohydrate capsules were first reported for *M. mycoides* about 50 years ago (Buttery and Plackett, 1960; Plackett and Buttery, 1964). Since then, studies have shown that the carbohydrates can either be secreted (EPS) or constitute a capsule (CPS). Both the CPS and EPS produced by *Mmm* consist of a β-(1→6)-galactofuranose homopolymer (Bertin *et al*., 2013; 2015), referred to as galactan in this study. Previous studies indicated that CPS in *Mycoplasma* spp. contributes to adhesion to epithelium (Tajima *et al*., 1982), has a down-regulating effect on alveolar macrophages (Almeida *et al*., 1992) and protects against complement (Bolland *et al*., 2012; Gaurivaud *et al*., 2014), but the biological role in pathogenicity in the '*Mycoplasma mycoides* cluster' (Cottew *et al*., 1987) has not been clarified. It is widely accepted that glycans, except zwitterionic carbohydrates, are unable to induce a $CD4^+$ T cell memory immune response (Comstock and Kasper, 2006), which could explain the lack of long-lasting immune responses after vaccination with the existing live vaccines. It is important to obtain more information on the role of carbohydrates with respect to host–pathogen interactions, before a glycoconjugate vaccine research pathway for *M. mycoides* should be followed. Therefore, attenuated strains represent promising tools that enable us to gauge the role of carbohydrates in pathogenicity.

Here we report the seamless deletion of the UDP-galactopyranose mutase encoding gene (*glf*) in *Mmc*, a model organism for its closest relative *Mmm*. We applied genome editing of the *Mmc* genome YCpMmyc1.1 (YCp-marked genome of the virulent strain GM12) in a yeast host using the TREC system (Noskov *et al*., 2010) to seamlessly delete *glf* before 'backtransplanting' the genome into *Mycoplasma capricolum* devoid of restriction enzyme (Lartigue *et al*., 2009). We then comparatively evaluated the GM12 wild type and YCpMmyc1.1-Δ*glf* strains for membrane integrity, adhesion to primary cells, growth rate and serum resistance.

Results

In silico *identification of gene clusters harbouring* glf *in members of the '*Mycoplasma mycoides *cluster'*

The biosynthesis of UDP-α-D-galactofuranose involves the enzymes UTP-glucose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase and UDP-galactopyranose mutase (encoded by *galU*, *galE* and *glf*, respectively) as depicted in Fig. 1A and Fig. S1. Glycosyltransferases use UDP-α-D-galactofuranose as the source of galactofuranose residues in the biosynthesis of the capsular polysaccharide (Richards and Lowary, 2009). However, it is unknown which of the several *in silico*-identified glycosyltransferases in *M. mycoides* is responsible for polymerisation.

A cluster of genes with a total size of about 10 kb consisting of *galU*, *galE*, a glycosyltransferase encoding gene *epsG* and *glf* was identified in *Mmc* strains GM12 and 95010 (Fig. 1B). The arrangement of these genes in *Mmc* strains GM12 and 95010 differed only with respect to the insertion site of an IS-element (Fig. 1B). In *Mmm* PG1 and Gladysdale, the entire cluster has a size of approximately 30 kb due to a tandem repeat of the gene cluster and the inclusion of another glycosyltransferase encoding gene *cps* (Fig. 1B). Although the annotation of

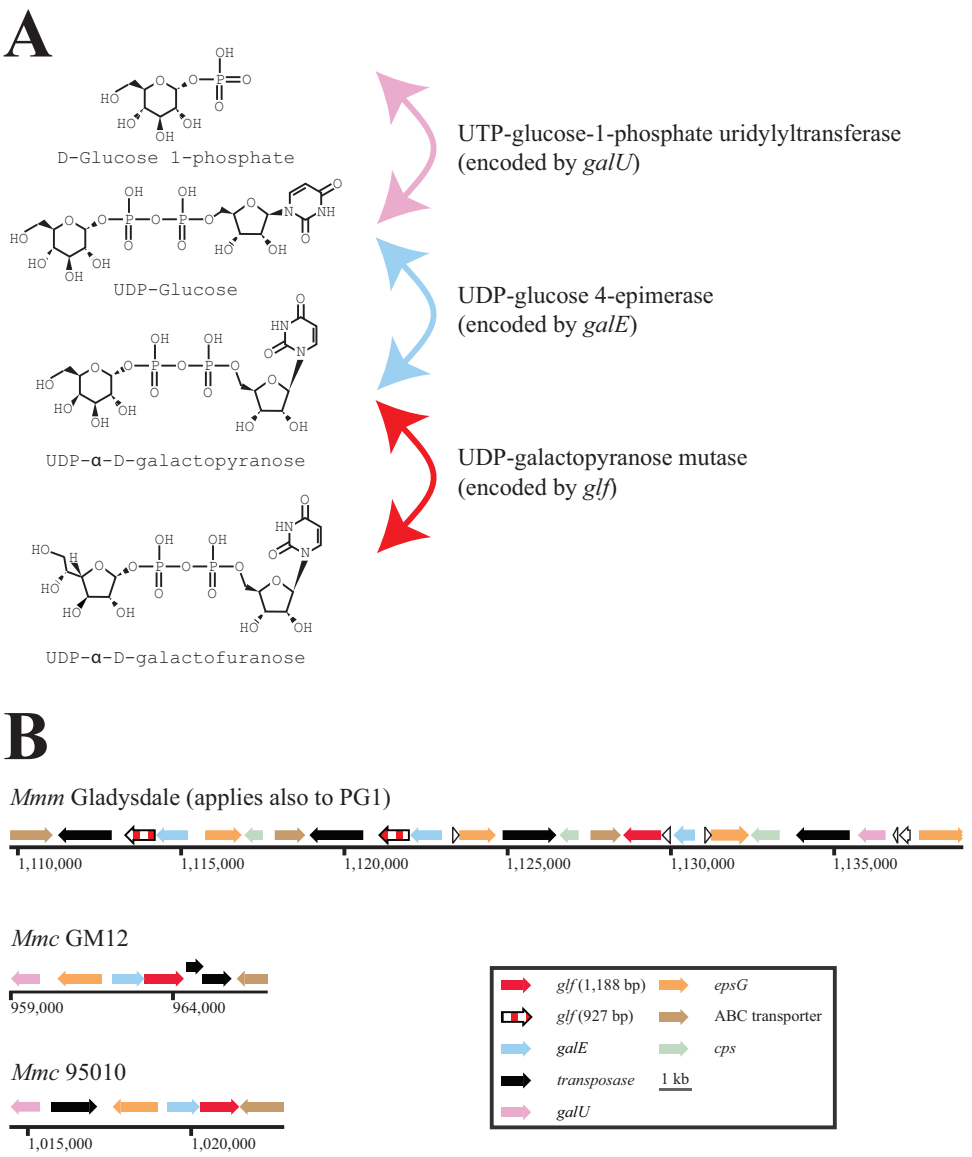

Fig. 1. *M. mycoides* subsp. *mycoides* and *M. mycoides* subsp. *capri* harbour either three copies (one full length functional and two truncated non-functional) or one copy (functional) of the UDP-galactopyranose mutase encoding gene *glf* respectively.
(A) Cartoon based on a KEGG analysis displaying the biosynthesis of UDP-α-D-galactofuranose starting from D-glucose-1-phosphate. (B) Cartoon representing the gene clusters harbouring the UDP galactopyranose mutase encoding *glf* in the two '*M. mycoides* cluster' members *M. mycoides* subsp. *mycoides* (strains Gladysdale and PG1) and *M. mycoides* subsp. *capri* (strains GM12 and 95010). Other genes within the locus are *galU* encoding UTP-glucose-1-phosphate uridylyltransferase, *epsG* encoding a glycosyltransferase and *galE* encoding UDP-glucose 4-epimerase). In *Mmm*, the locus also contains the *cps* encoding a glycosyltransferase.

the genomes of *Mmm* PG1 and Gladysdale reveals the presence of three copies of the gene *glf*, two of them have a single nucleotide deletion leading to a frameshift resulting in premature stop codons and truncated reading frames of 927 bp instead of full-length 1,188 bp.

Because the major component of both the capsule and excreted polysaccharides in *Mmm* and *Mmc* has been shown to be galactofuranose (Plackett and Buttery, 1964; Bertin *et al.*, 2013; 2015), we focused our work on UDP-galactopyranose mutase encoded by *glf*, which converts UDP-α-D-galactopyranose into UDP-α-D-galactofuranose. Extensive searches for *glf* in other species of the '*Mycoplasma mycoides* cluster' did not reveal the presence of this gene in the genomes of *M. capricolum* subsp. *capricolum (Mcc)*, *M. capricolum* subsp. *capripneumniae* (*Mccp)* or *M. leachii*.

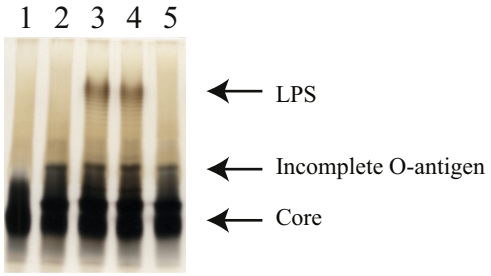

Fig. 2. Results of the *in vivo* complementation assays in *Escherichia coli* to verify functionality of the *glf* encoded enzymes. *E. coli* strain MFF1 (*wbbL- glf-*) produces complete LPS in its O antigen repeating units only when complemented with *wbbL* and a functional UDP-galactopyranose mutase (*glf*) homologue. LPS preparations were analysed by SDS-PAGE followed by silver staining of LPS. Lane 1, MFF1 *E. coli* strain alone, no O-antigen is produced. Lane 2, MFF1 *E. coli* strain complemented with *wbbL* and empty pEXT20 vector, only incomplete O-antigen is produced. Lane 3, MFF1 *E. coli* strain complemented with *wbbL* and the pEXT20 vector containing *glf* from *Mmm* strain Gladysdale (MMS_A1075 spanning 1,188 bp), complete LPS is produced. Lane 4, MFF1 *E. coli* strain complemented with *wbbL* and the pEXT20 vector containing *glf* from *Mmc* strain GM12 (MMCAP1_0814 spanning 1,188 bp), complete LPS in produced. Lane 5, MFF1 *E. coli* strain complemented with *wbbL* and the pEXT20 vector containing *glf* from *Mmm* strain Gladysdale (MMS_A1062 spanning 927 bp), only incomplete O-antigen is produced.

Functional testing of Mmc *and* Mmm *UDP-galactopyranose mutases using an* E. coli*-based complementation assay*

Escherichia coli strains of the serotype O16 contain a terminal galactofuranose residue linked to rhamnose in their O antigen repeating units. The laboratory strain W3110 is a derivative of O16 with a mutation in the *wbbL* gene, involved in the addition of the second sugar, rhamnose, to the O antigen subunit (Stevenson *et al*., 1994). *E. coli* strain MFF1 (*wbbL- glf-*) is a derivative of W3110 that lacks both a functional *wbbL* and a *glf* gene (Feldman *et al*., 1999). When complemented with the rhamnosyl transferase WbbL, MFF1 produces an incomplete O antigen subunit that lacks the terminal galactofuranose residue. If this strain is complemented with both, *wbbL* and a functional UDP-galactopyranose mutase (*glf*) homologue, synthesis of full-length O antigen is restored and the LPS carries a full O 16 antigen (Poulin *et al*., 2010).

To test if *glf* homologues from *Mmc* and *Mmm* encode a functional UDP-galactopyranose mutase, strain MFF1 was complemented with WbbL and plasmids containing one of the *glf* genes (full-length or truncated from *Mmm* Gladysdale and *Mmc* GM12). LPS preparations were analysed by SDS-PAGE followed by silver staining (Fig. 2). When MFF1 was complemented with WbbL, a single incomplete O antigen subunit is transferred to lipid A (lane 2). Introduction of full-length *glf* genes from *Mmm* (lane 3) and *Mmc* (lane 4) into MFF1 WbbL$^+$ resulted in the production of fully polymerized O antigen. The truncated *glf* gene from *Mmm* (lane 5) was unable to restore O antigen synthesis. These results showed that *Mmc* and *Mmm* contain functional *glf* genes, together with truncated non-functional *glf* homologues in *Mmm* strains.

Presence of galactofuranose-containing galactan in Mycoplasma mycoides *strains*

To confirm the presence of galactofuranose-containing galactan in *M. mycoides* strains, we required specific antibodies for galactan made of β-(1→6)-galactofuranose. Among individual hybridoma cells screened by ELISA technique, hybridoma AMMY10 produced an IgM antibody that bound both to whole *Mmm* Afadé and purified *Mmm* Afadé capsular polysaccharide. A high molecular weight pattern, typical of large polysaccharides, was shown in a western blot using the AMMY10 monoclonal antibody, similar to the pattern stained by the carbohydrate specific periodic acid-Schiff stain (PAS). The isolated carbohydrates were free of proteins as shown by Coomassie staining and by dot blot analysis using a rabbit polyclonal antibody raised against full-length recombinant *Mmm* elongation factor Tu. As both *Mmm* and *Mmc* produce the same galactan, a β-(1→6)-galactofuranose homopolymer (Bertin *et al*., 2013; 2015), and AMMY10 recognised purified carbohydrates from *Mmm* and *Mmc*, we concluded that this monoclonal antibody was specific for galactan consisting of β-(1→6)-galactofuranose and proposed to use it in subsequent analyses.

Expression of the galactofuranose-containing polysaccharide was monitored by dot blot analysis of whole cell lysates from 14 *Mmm* and 15 *Mmc* strains using the monoclonal antibody AMMY10 (Fig. S2). As expected, all *Mmm* strains reacted with the antibody. The *M. capricolum* subsp. *capricolum* control strain ATCC 27343 was not recognised, which is in alignment with the absence of the gene *glf* in this strain. Out of the 15 *Mmc* strains tested, 13 reacted with the antibody. Only the two *Mmc* strains PG3 and CapriL did not react with the mAb. A recent study has also shown that *Mmc* PG3 and CapriL do not contain galactofuranose-containing galactan (Bertin *et al*., 2015). Therefore, this analysis additionally confirms the specificity of the monoclonal antibody AMMY10 to recognise galactofuranose-containing galactan.

Generation of a Mmc glf *knock-out mutant*

After transformation of *Saccharomyces cerevisiae* containing the YCpMmyc1.1 genome with TREC (tandem

repeat coupled with endonuclease cleavage) constructs specific for deletion of the *glf* gene, integration of the construct at the correct locus was confirmed via PCR. The TREC construct was removed using counter selection, and a seamless deletion of *glf* was confirmed by sequencing. As a result of the primer design for deletion of the gene, the deletion extended from nucleotide 18 of the *glf* gene to 15 nucleotides downstream of the *glf* stop codon. The genome integrity after deletion was first confirmed via a multiplex PCR employing eight primer pairs evenly spread out over the *Mmc* genome. Amplicons of the expected size from all primer pairs confirmed that the genome had not undergone major recombination events during propagation in yeast cells (Fig. S3A). Additionally, the double band profile (~670 kb and ~420 kb) visible on pulsed-field gel electrophoresis (PFGE) confirmed the completeness of the YCpMmyc1.1-Δ*glf* genome in two yeast clones (Fig. S3B).

The entire genome with the deletion of the *glf* gene, YCpMmyc1.1-Δ*glf*, was then transplanted into recipient cells *Mcc* RE(-). The integrity of the transplanted genome in the resulting *Mmc* clone was confirmed using the multiplex PCR described above, and the seamless deletion of *glf* was again confirmed using PCR (Fig. S4).

*Full genome sequencing of the transplanted YCpMmyc1.1-*Δglf

To ensure the genome had not undergone any rearrangements during the maintenance of the genome in yeast, we sequenced the whole genome using PacBio technology. *De novo* assembly and polishing resulted in one single contig of 1 085 764 bp (GenBank Accession No. CP012387). Mapping the genomic sequence to that of a previously sequenced mutant GM12 clone, YCpMmyc1.1-ΔtypeIIIres (GenBank Accession no. CP001668) (Lartigue *et al*., 2009) revealed two expected rearrangements: the deletion of the *glf* gene in YCpMmyc1.1-Δ*glf*, and the deletion of the gene encoding a type III restriction enzyme in the reference genome. Three additional changes were identified in YCpMmyc1.1-Δ*glf*, which we confirmed by Sanger sequencing. These were (i) A deletion of TATA in a stretch of 12 TA repeats in an intergenic region between two putative lipoproteins (MMCAP2_0900 and MMCAP2_0901); (ii) A SNP (C to T transition) in MMCAP2_0183 encoding a putative maltose ABC transporter permease, resulting in an amino acid change from threonine to isoleucine at amino acid position 837 (out of 846); (iii) A deletion of an A nucleotide in a stretch of six As at the 3′ end of *galE* (MMCAP2_0813), the gene located upstream of *glf*. The encoded enzyme UDP-glucose-4-epimerase forms part of the same pathway as UDP-galactopyranose mutase (encoded by *glf*) (Fig. 1). This single nucleotide deletion lead to a frameshift where the two most C-terminal amino acids (Asn-Lys) of the wild-type UDP-glucose 4-epimerase are replaced by a stretch of 27 amino acids before a stop codon.

Functional testing of the mutated UDP-glucose-4-epimerase using an E. coli*-based complementation assay*

Our sequencing data demonstrated that in the transplanted YCpMmyc1.1-Δ*glf*, an undesired mutation in the *galE* gene affecting the stop codon resulted in a protein containing a C-terminal extension of 27 amino acids. Since UDP-α-D-galactopyranose is required as a precursor for the synthesis of UDP-α-D-galactofuranose by UDP-galactopyranose mutase (Fig. 1), we explored if the C-terminal extension affected UDP-glucose-4-epimerase activity. Because UDP-α-D-galactose is also required for the synthesis of O7-antigen in *E. coli*, we tested the activity of the *galE* mutant variant by introducing the wild type or mutant version of the mycoplasma *galE* gene in the *E. coli* DH10B strain (mutant for *galE*), together with the plasmid pJHCV32 that contains the necessary genes for the synthesis of the O7 antigen, excepting *galE* (Valvano and Crosa, 1989). We observed that both the wild type and the mutant *galE* genes are able to complement the DH10B strain, allowing the expression of the O7 antigen (Fig. S5), indicating that the activity of the protein product of the *galE* gene is not affected in the mutant variant. As a positive control for O7 antigen expression, we introduced the plasmid pJHCV32 in the *E. coli* W3110 strain, which contains an active copy of the *galE* gene. This experiment demonstrated that the longer *galE* encoded in YCpMmyc1.1-Δ*glf* was functional and is not likely to cause any phenotype observed for YCpMmyc1.1-Δ*glf*.

Absence of galactofuranose-containing glycans in the Mmc glf *knock-out mutant*

The monoclonal antibody AMMY10, recognising *Mmm* and *Mmc* capsular polysaccharides was tested for its reactivity with whole cell lysates of *Mmc* GM12 wild type, *Mmc* YCpMmyc1.1 and *Mmc* YCpMmyc1.1-Δ*glf* (Fig. 3A). As expected, the antibody reacted with the parental strains GM12 and YCpMmyc1.1, but not with YCpMmyc1.1-Δ*glf*, confirming the absence of galactofuranose-containing galactan in the Δ*glf* mutant. PAS and Coomassie staining are also shown for comparison: PAS staining (Fig. 3B) shows the same pattern of staining as the AMMY10 antibody staining in the western blot for GM12 and YCpMmyc1.1. and YCpMmyc1.1-Δ*glf*. The Coomassie stain is shown as a loading control (Fig. 3C).

Additionally, total carbohydrates from GM12, YCpMmyc1.1 and YCpMmyc1.1-Δ*glf* were isolated from both bacteria and culture medium, to include any polysac-

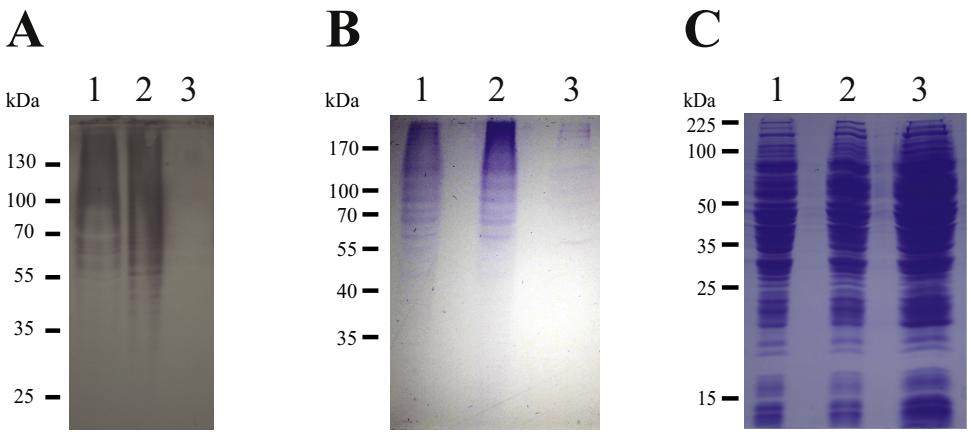

Fig. 3. YCpMmyc1.1-Δ*glf* lacks the galactofuranose-containing galactan – SDS-PAGE.
Whole bacterial lysates were analysed by SDS-PAGE to an equivalent of 10 μg (A) or 20 μg (B and C) of protein each, either transferred to nitrocellulose membranes for western analysis using the AMMY10 monoclonal antibody (A) or stained by periodic acid-Schiff stain (PAS) (B) or Coomassie staining (C). Lane 1: GM12 wild type; Lane 2: YCpMmyc1.1; Lane 3: YCpMmyc1.1-Δ*glf*.

charides derived from either capsule or EPS. The isolated carbohydrates were analysed by SDS-PAGE, followed by western blot analysis employing the AMMY10 antibody, and by PAS and Coomassie staining (Fig. S6). The Coomassie staining and dot blot analysis for full-length recombinant *Mmm* elongation factor Tu confirmed the absence of protein in the preparation. Taken together, the western blot and PAS analyses confirmed that YCpMmyc1.1-Δ*glf* does not produce a galactofuranose-containing galactan.

Absence of galactofuranose-containing glycans in the mutant was also confirmed using immunoelectron microscopy (Fig. 4). Polysaccharides recognised by AMMY10 were localised at the cell periphery, on extracellular material or in close vicinity to the cells. In the mutant YCpMmyc1.1-Δ*glf* no binding of the antibody could be

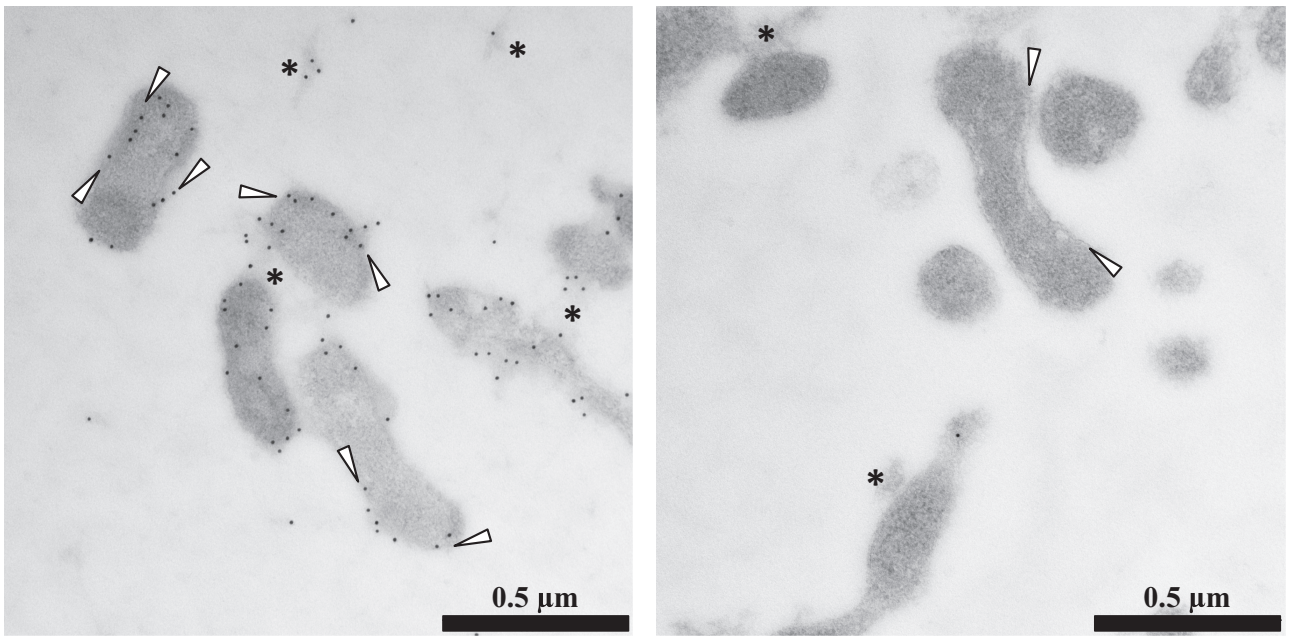

Fig. 4. YCpMmyc1.1-Δ*glf* lacks the galactofuranose-containing galactan – electron microscopy.
Immunoelectron microscopy of *M. mycoides* using the AMMY10 antibody. Left: GM12; Right: YCpMmyc1.1-Δ*glf*. Antibodies react with the cell periphery of the wild type (arrowheads) and also some extracellular material (asterisks), while in the mutant no reaction occurred.

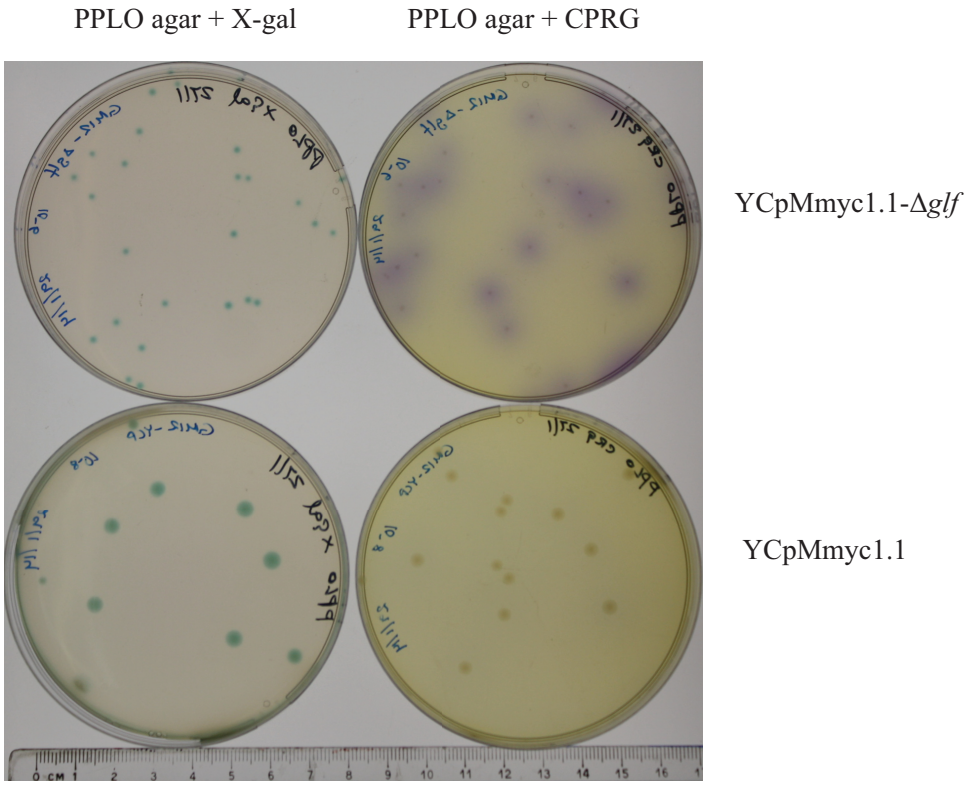

Fig. 5. Galactofuranose-containing glycans contributes to membrane integrity in *Mycoplasma mycoides*.
The left panel shows YCpMmyc1.1-Δ*glf* and YCpMmyc1.1, both containing the *lacZ* gene growing media containing the membrane permeable β-galactosidase substrate X-gal. As expected colonies show the typical turquoise colouring in both strains. The right panel shows growth on the membrane impermeable β-galactosidase substrate CPRG (upper right). Only the YCpMmyc1.1-Δ*glf* lacking galactofuranose-containing glycans show colouring of and around the colonies, whereas the control does not show any colour reactions.

detected apart from a few single gold beads (Fig. 4B) that were also observed in both strains when using only the secondary antibody (Fig. S7).

Deletion of the glf *gene reduces the membrane integrity of* Mmc

Capsular polysaccharides and cell walls contribute to cell integrity in bacteria. As mycoplasma do not contain a cell wall, we hypothesised that deletion of CPS content was likely to compromise membrane integrity of the cell. We used an assay recently described to screen for bacterial envelope biogenesis mutants in *E. coli* (Paradis-Bleau *et al.*, 2014), based on the assumption that mutants deficient in building blocks/pathways for envelope biogenesis either lyse more frequently and/or are more permeable to small hydrophobic molecules. This assay employed CPRG, a cell membrane impermeable substrate for β-galactosidase, compared with X-gal, a cell membrane permeable substrate for β-galactosidase, which provides a blue/white colony screening system. In our case, *lacZ* is under the constitutive spiralin promoter (Lartigue *et al.*, 2002) in the YCpMmyc1.1 and YCpMmyc1.1-Δ*glf* clones (Lartigue *et al.*, 2009). To confirm expression of *lacZ*, the clones were grown on agar containing either X-gal (cell membrane permeable) or CPRG. Both clones developed blue colonies on the X-gal agar, but only YCpMmyc1.1-Δ*glf* showed hydrolysis of CPRG, indicating that this strain is more permeable to this compound (Fig. 5). We concluded that the galactofuranose-containing polysaccharide contributes to membrane integrity of the mycoplasma tested, and lack thereof leads to leakiness of the mycoplasma cell. Another explanation would be that the enzyme UDP-galactopyranose mutase directly affects cell membrane integrity if it would be an integral part of the membrane, but its membrane localisation in the mycoplasma cell has not been reported yet (Krasteva *et al.*, 2014).

Evaluation of Mmc glf *knock-out mutant generation time*

We studied the growth rates of GM12, YCpMmyc1.1 and YCpMmyc1.1-Δ*glf*. We did not observe a significant difference between GM12 and YCpMmyc1.1, which exhib-

ited generation times of 56 and 57 min respectively. However, YCpMmyc1.1-Δ*glf* showed a marked decrease in growth rate, with a generation time of 89 min, also indicated by a smaller colony size (Fig. 3). Thus, a lack of UDP-galactopyranose mutase and therefore of galactofuranose-containing glycans leads to an increased generation time and reduced colony size.

Impact of galactofuranose-containing galactan on cell adhesion

We tested if the galactofuranose-containing glycan plays a role in adhesion to small ruminant cell line LSM 192 (Zimmermann *et al*., 2010). Saturation of adhesion was obtained for wild-type strain GM12 and YCpMmyc1.1 at a multiplicity of infection (moi) of 1,600 mycoplasma cells per LSM 192 cell and for YCpMmyc1.1-Δ*glf* at an moi of 25 600 per LSM 192. At this moi, GM12 adhered with an average of 808 mycoplasmas cells per LSM 192 cell (considered as wild-type adherence standard = 100%) and its derivative YCpMmyc1.1 with an average of 178 mycoplasma cells per LSM 192 cell. The UDP-galactopyranose mutase deficient mutant YCpMmyc1.1-Δ*glf* in contrast showed an approximately 15-fold higher adhesion compared with the wild-type strain and 70-fold higher adhesion rate compared with the isogenic parent YCpMmyc1.1 resulting in 12 486 mycoplasma cells per LSM 192 cell (Fig. 6). The results are significant ($P = 0.0036$) according to the non-parametric Kruskal–Wallis test. We concluded that lack of UDP-galactopyranose mutase and the galactofuranose-containing galactan leads to increased adhesion to host cells.

Effect of galactofuranose-containing galactan on serum resistance

Evasion of killing by complement is required for colonisation of many bacterial pathogens. Bacterial polysaccharides, such as capsule and O antigen, have often been implicated in resistance to complement killing, including for *Mycoplasma* spp. (Bolland *et al*., 2012; Osawa *et al*., 2013; Gaurivaud *et al*., 2014). For this reason, we investigated if the presence of the galactofuranose-containing galactan has an effect on serum resistance in *Mmc* GM12. Contrary to our predictions, the mutant strain YCpMmyc1.1-Δ*glf* lacking the galactofuranose-containing galactan was resistant to serum killing when using fresh goat serum (Fig. 7) while GM12 and YCpMmyc1.1 were highly sensitive. All three strains survived treatment with heat-inactivated serum.

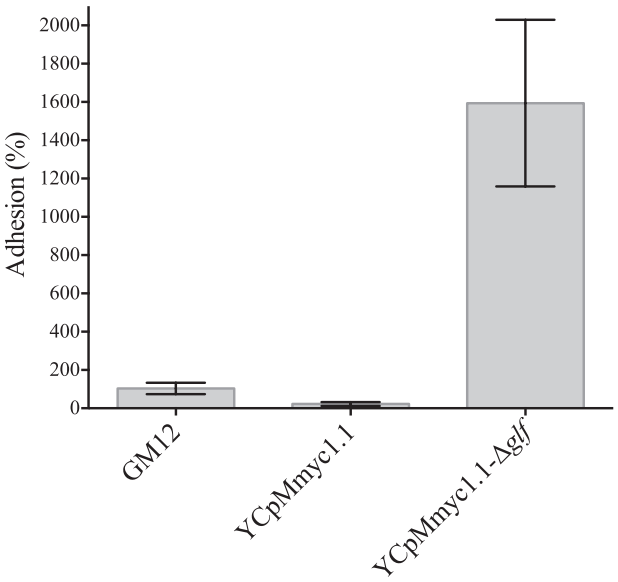

Fig. 6. Galactofuranose-containing galactan conceals adhesion molecules in *Mycoplasma mycoides*.
The histogram displays the results of an adhesion assay of wild-type GM12 and its derivative mutant strains with primary lamb cells from carpal joint synovial tissue (LSM 192). Adhesion was determined using incorporation of [U-^{14}C] palmitic acid in mycoplasma cells. The adhesion values of the strain GM12 were used as standard (100%), and the adhesion values of YCpMmyc1.1 and YCpMmyc1.1-Δ*glf* were compared against that standard. Standard error is shown.

Discussion

Specific protective antibody responses to mycoplasma proteins have not been identified for diseases caused by *M. mycoides* such as CBPP. The chronic phase of CBPP associated with the presence of viable mycoplasma and the inability to induce antibodies that neutralise virulence factors such as l-α-glycerol-3-phosphate oxidase, suggesting the existence of immune evasion mechanisms by mycoplasma (Mulongo *et al*., 2013; Schieck *et al*., 2014). In this study we investigated the role of polysaccharides of *M. mycoides* in the subspecies *capri* (*Mmc*), which is phylogenetically the closest relative of *M. mycoides* subsp. *mycoides* (*Mmm*). It has been shown that *M. mycoides* produces carbohydrates consisting of galactofuranose residues, which are either present in the capsule or excreted into the medium (Bertin *et al*., 2013; 2015; Gaurivaud *et al*., 2014). Our data utilising an anti-galactofuranose monoclonal antibody confirmed the presence of this galactan in *Mmc* GM12 and other *M. mycoides* strains (Fig. S2). To gain a better understanding on the role of the galactofuranose-containing galactan in the biology of *M. mycoides*, we investigated the *glf* gene in *Mmm* and *Mmc*, predicted to encode UDP-galactopyranose mutase, the enzyme that converts UDP-α-D-galactopyranose into UDP-α-D-galactofuranose. We showed that both subspecies of *M. mycoides* contain only one copy of the *glf* gene that encodes a functional UDP-galactopyranose mutase. *Mmm* PG1 and Gladysdale

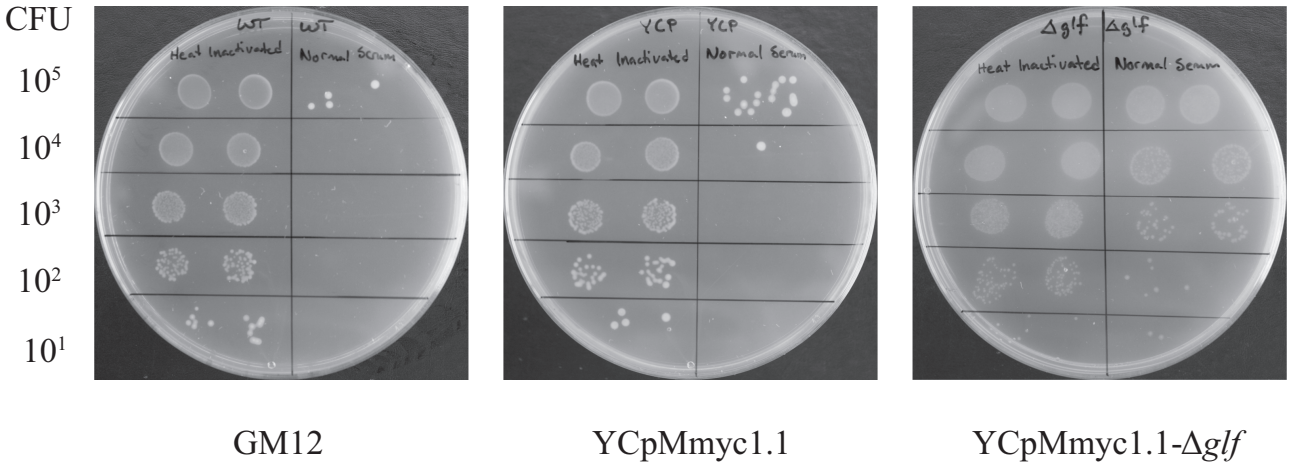

Fig. 7. Lack of galactofuranose-containing galactan renders GM12 resistant to killing by caprine serum.
Survival of the three strains GM12, YCpMmyc1.1 and YCpMmyc1.1-Δ*glf* after 30 min incubation at 37°C with 50% fresh goat serum is shown. Serial dilutions from 10^5-10 cfu were spotted in duplicates, either after incubation with heat-inactivated serum (left on each plate) or with untreated normal serum (right on each plate).

harbour two additional but truncated copies of *glf*, which do not code for a functional UDP-galactopyranose mutase (Fig. 2). A frameshift at the same nucleotide position within the two truncated genes suggests a duplication event. Whether the encoded truncated proteins are expressed *in vivo* and play a different role remains unknown. Interestingly, proteomic studies have suggested that the enzyme UDP-galactopyranose mutase is immunogenic in *Mmm* (Jores *et al*., 2009), which might be attributed to a possible high abundance of the protein.

We confirmed the presence of a galactofuranose polysaccharide in all *Mmm* and most *Mmc* strains tested. Two out of fifteen *Mmc* strains did not react with the galactan-specific antibody. This is in accordance with recently published data indicating that members of the *Mmc* 'capri' serovar do not produce galactan (Bertin *et al*., 2015), as opposed to the *Mmc* 'Large Colony' serovar (Manso-Silvan *et al*., 2009).

Subsequently, we deleted the *glf* gene in the highly virulent small ruminant pathogen *Mmc* GM12 (DaMassa *et al*., 1983; 1986) employing the recently developed genome transplantation technique with editing of the *Mmc* genome within yeast as an intermediary host (Lartigue *et al*., 2007; 2009). This technique has rendered *Mmc* a model organism that can be genetically modified in a way that has not yet been achieved for other *Mycoplasma* species. Additionally, the genome of *Mmc* contains a single copy of the gene *glf* and its gene cluster, which simplifies mutagenesis approaches and allows unambiguous results given the known tandem repeats of the *glf* containing cluster in *Mmm* (Fig. 1) (Westberg *et al*., 2004). As expected by our KEGG-based *in silico* analysis, the *glf*-deficient strain no longer contains galactofuranose-containing glycans as shown by immunochemical experiments involving the murine monoclonal antibody AMMY10 that recognises *M. mycoides* galactofuranose polysaccharides.

Our study provides evidence that UDP-galactopyranose mutase or more likely its downstream product, the galactofuranose-containing galactan contribute to membrane integrity, as shown by the β-galactosidase assays (Fig. 5). The slower growth rate of the *glf*-mutant strain may be a result of this 'leaky membrane' phenotype. We hypothesise that this increased generation time is caused by disequilibrium of nutrients and salts within the mycoplasma cell, which leads to the need of more energy-dependent efforts to maintain basic homeostasis to keep the cell machinery functioning. Another possibility is that the reduced growth rate is caused by accumulation of a toxic intermediate or a structural role of galactofuranose in modification of glycolipids/glycoproteins. As glycoglycerolipids are key structural membrane elements in *Mycoplasma* spp. (Andres *et al*., 2011), our data indicate that galactofuranose-containing galactan is a component of membrane glycoglycerolipids in *M. mycoides*. Targeting the galactan in vaccine or antimicrobial development efforts is a promising avenue to pursue as this is likely to have an impact on the viability of *Mycoplasma* spp. in the host.

Another prominent finding of our study is the lower adherence of the wild-type strain to carpal joint synovial cells compared with the *glf*-deletion mutant. Thus, the galactofuranose-containing glycans appear to conceal the adhesion molecules of the wild-type strain, which has also been described for other pathogens (Feng *et al*., 2012). This phenomenon can be interpreted as a kind of trade

off. The galactofuranose polymer covers adhesins that consequently may be less exposed to the humoral immune system. Additionally, it is known that *M. mycoides* infections are often associated with lower respiratory tract infections (Thiaucourt and Bolske, 1996; Caswell and Williams, 2007), and a less adhesive bacterium is likely to travel deeper into the target region of the lung tissue. This reduces the likelihood of colonising the upper respiratory tract (trachea or bronchial tract) and being removed by the mucocilliary clearance.

Although capsules are reported to be responsible for resistance to killing by complement in other bacterial species (Miajlovic and Smith, 2014), the *glf* deletion mutant had higher serum resistance compared with its parental strain. Previous studies looking at *Mmm* and *M. pulmonis* suggest that mycoplasma polysaccharides protect against complement (Bolland *et al*., 2012; Gaurivaud *et al*., 2014). The study on *Mmm* investigated clones of *Mmm* strain Afadé that either secrete galactofuranose-containing glycans into the medium or have a galactofuranose-containing galactan bound to the cell membrane. The study of Gaurivaud *et al*. (2014) is based on *Mmm* strain Afadé wild-type clones (and not a galactofuranose negative mutant). Therefore, a comparison of their studies with those reported here is difficult. One possible explanation for our finding is that an important trigger for activation of the complement cascade occurs through galactofuranose residue-binding lectins. Humans have been reported to possess human intelectin (hIntL), a lectin that specifically binds to galactofuranose-containing carbohydrates (Tsuji *et al*., 2001). Goats have been reported to encode carbohydrate binding lectin-encoding genes, but a characterisation of caprine lectins has not yet been performed (Barik *et al*., 2014). In the absence of galactofuranose, the complement system could function less efficiently due to the absence of sufficient target molecules for activation. A second possible explanation is that mycoplasma might express a complement-degrading factor as reported in other pathogens (Rambach *et al*., 2010), which is more exposed in the *glf* mutant. The enhanced exposure of surface mycoplasma molecules such as lipoproteins (Chambaud *et al*., 1999; Browning *et al*., 2011), glycolipids or glycoproteins (Jordan *et al*., 2013), might contribute to protection against serum killing. Alternatively, the phenotypes observed in this study, including altered membrane integrity and increased serum resistance and host cell adhesion, could theoretically be correlated to the absence of the protein UDP-galactopyranose mutase instead of the galactofuranose-containing glycans. This is rather unlikely, as UDP-galactopyranose mutase is not part of the *in vitro* surface proteome (Krasteva *et al*., 2014) in nine closely related *Mmm* strains tested. It is also possible that an accumulation of galactopyranose inside the cell could result in leaky cells; however, that this could lead to increased adhesion or increased serum killing resistance is not very likely.

Full genome sequencing of the YCpMmyc1.1-Δ*glf* clone identified three mutations compared with the parental strain (not nosidering the genome edits), each of which could theoretically cause the phenotypes described here. The deletion of TATA occurring in a region upstream of a putative lipoprotein (Locus tag: MMCAP2_0900) that might be involved in phase variable expression, is not very likely to cause the described growth rate reduction, decreased membrane integrity, increased adhesion to host cells or increased serum resistance. A single amino acid change from threonine to isoleucine in a putative maltose ABC transporter permease (MMCAP2_0183) is also not likely to cause the phenotypes described here. Because the mutated *galE* gene encodes a functional UDP-glucose 4-epimerase, it can be excluded as the cause of the phenotypes observed.

Collectively, our *in vitro* assays point towards an important role of galactofuranose-containing glycans in the homeostasis of *M. mycoides* and in the host–pathogen interactions. We will try to decipher the enzyme pathways involved in capsule synthesis in the future to identify targets for vaccine and therapeutic products using synthetic genomics and genome editing techniques as shown here. Future experiments should involve *in vivo* studies to elucidate the role of galactofuranose-containing glycans in pathogenicity and the potential of this capsule deficient strain in immunisation experiments.

Experimental procedures

Yeast and Mycoplasma *strains used in this study and culture conditions*

Saccharomyces cerevisiae strain W303a containing the marked *Mmc* genome YCpMmyc1.1 (Lartigue *et al*., 2009; Benders *et al*., 2010) and its derivative YCpMmyc1.1-Δ*glf* were grown on synthetic minimal medium containing glucose (SD) either lacking histidine, or histidine and uracil, or lacking histidine and supplemented with 1 mg ml^{-1} 5-fluoroorotic acid (5-FOA, Carl Roth, Germany) (Kouprina and Larionov, 2008; Noskov *et al*., 2010).

All *Mycoplasma* strains *Mmc*: GM12 wild type, YCpMmyc1.1, YCpMmyc1.1-Δ*glf*, My325, G1283.94, G1313.94, 7302, 7730, Kombolcha, 9096.C9415, C260/4, PG3, CapriL, Y-goat, D2482, Wi18079 and 95010; *Mmm*: Afadé, Tan8, Matapi, T1/44, B237, PO2, Madigwan, Fatick, Shawawa, Astercous, L2, C11, V5 and N6; and *Mcc*: ATCC 27343 (California Kid) were grown at 37°C in PPLO medium (Difco, Cat no. 255420) supplemented with 20% heat-inactivated horse serum (Sigma, Cat. No. H1138), 0.5% glucose, 0.03% penicillin G, 20 mg ml^{-1} thallium acetate and 0.9 g l^{-1} yeast extract, unless otherwise stated, and in SP4 medium with or without 10 μg ml^{-1} tetracycline as described elsewhere (Lartigue *et al*., 2009).

In silico *identification of UDP-galactopyranose mutase encoding genes (*glf*) and associated genes in members of the '*Mycoplasma mycoides *cluster'*

The *in silico* analysis of the galactose pathway in *Mycoplasma mycoides* was carried out using the KEGG database (Kanehisa *et al*., 2014) (Fig. S1). The genomes of the fully sequenced '*M. mycoides* cluster' strains *Mmc* GM12, (GenBank accession number CP001621.1), *Mmc* 95010 (GenBank accession number NC_015431.1), *Mmm* PG1^T (GenBank accession number NC_005364.2), *Mmm* Gladysdale (GenBank accession number CP002107.1), *Mcc* ATCC27343 (GenBank accession number NC_007633.1); *Mccp* M1601 (GenBank accession number CM001150.1); *M. leachii* 99/014/6 (GenBank accession number FR668087.1); *M. leachii* PG50 (GenBank accession number NC_014751.1) were screened for the gene cluster containing the predicted UDP-galactopyranose mutase encoding gene *glf* using the BLAST algorithms.

Testing the functionality of M. mycoides *UDP-galactopyranose mutase encoded by* glf *via a complementation assay in* Escherichia coli

Homologues of the gene *glf* found in *Mmc* GM12 (MMCAP1_0814, spanning 1,188 bp) and in *Mmm* Gladysdale (MMS_A1075, spanning 1,188 bp and MMS_A1062, spanning 927 bp) were synthesised using *E. coli* codon optimisation (GenScript), and placed in pUC57 multipurpose cloning vector; *glf* candidate genes were subcloned with a C-terminal His tag in the *E. coli* expression vector pEXT20 (Dykxhoorn *et al*., 1996) using EcoRI and HindIII sites. The synthesised codon optimised nucleotide sequences can be found in Table S1.

Escherichia coli strain MFF1 (*wbbL- glf-*) (Feldman *et al*., 1999) was complemented with *wbbL* and pEXT20 containing the *glf* homologues under control of the tac promoter. Only after complementation with a functional UDP-galactopyranose mutase will synthesis of full-length O antigen be restored. LPS preparations were done as described previously (Marolda *et al*., 2006). Briefly, cultures were diluted to an optic density of 3.0 per ml (600 nm wavelength), and 1 ml of each culture was harvested by centrifugation, then resuspended in 150 µl of lysis buffer [2% SDS, 4% β-mercaptoethanol, 10% glycerol, 0.5 M Tris (pH 6.8)], and incubated at 100°C for 10 min. After addition of 2 µl of proteinase K (20 mg ml^{-1}), samples were incubated at 60°C for 2 h. To this solution was added 150 µl of hot phenol, and samples were incubated at 70°C for 15 min, followed by 10 min on ice. After centrifugation, the aqueous phase was mixed with 250 µl of ethanol and centrifuged, and the precipitated LPS was dried at room temperature. LPS samples corresponding to an absorbance of 0.45 at 600 nm were separated by SDS-PAGE, and LPS silver staining was performed as described elsewhere (Tsai and Frasch, 1982).

Ethics statement

All protocols of this study were designed and performed in strict accordance with the Kenyan legislation for animal experimentation and were approved by the ILRI animal care and use committee (reference numbers ILRI2010.06.21 and ILRI2014.26).

Monoclonal antibody production

Monoclonal antibody AMMY10 was obtained from a fusion of spleen cells from a mouse immunised against whole, sonicated *Mmm* strain Afadé. The strain was cultured as described above to a density of 10^8 colony-forming units (cfus) ml^{-1}, and centrifuged at 5,000 *g* for 20 min. The bacteria were suspended in PBS, sonicated three times for 2 min and mixed with an equal volume of TiterMax Gold (CytRx Corporation, Georgia, USA). A final amount of antigen equivalent to 10^9 mycoplasma cells was injected intraperitoneally into a 6 month old Balb/C mouse. A booster injection with the same preparation and amount was given 4 weeks after the initial immunisation. A final injection was given 6 weeks later, but this time without adjuvant. Four days later the mouse was sacrificed, and its spleen cells were isolated. A fusion of the spleen cells with X63.Ag8.653 myeloma cells was carried out as described previously (Naessens *et al*., 1985). The antibodies produced by individual hybridoma cells were screened using ELISA technique, first for recognition of whole cell lysates of *Mmm* Afadé, and then for recognition of capsular polysaccharides. Hybridoma AMMY10 produced an IgM antibody that bound to whole mycoplasma and purified capsular polysaccharide.

Detection of the galactofuranose-containing galactan in M. mycoides *strains*

Cultures of 15 *Mmc*, 1 *Mcc* and 14 *Mmm* strains were grown in PPLO media until log phase. The bacteria were harvested via centrifugation at 3,000 × *g* for 30 min and washed with PBS. Protein concentration was determined using the BCA Protein Assay Reagent kit (Pierce, Cat. no. 23225), following manufacturer's instructions. Samples were blotted onto Protran BA85 nitrocellulose membrane (Schleicher & Schuell) at an equivalent of 1 µg total protein per strain, and let dry at room temperature overnight. The murine monoclonal antibody AMMY10 was incubated with the dot blots at a dilution of 1:200 at room temperature for 1 h. Following five washing steps of submerging the membranes in fresh PBS containing 0.1% Tween20 (PBS-T) for 10 min each, alkaline phosphatase conjugated α-mouse IgM (dilution 1:30,000, Sigma, Cat. no. A9688) was added, and the blots were incubated at room temperature for 1 h. After five washes (PBS-T, 10 min each), the blots were stained with NBT/BCIP (Promega, Cat.no. S380c/S381c) solution and developed at room temperature for 10–15 min.

Deletion of the glf *gene in* Mycoplasma mycoides *subsp. capri strain GM12*

Saccharomyces cerevisiae W303a harbouring the *Mmc* GM12 YCpMmyc1.1 genome (Lartigue *et al*., 2009; Benders *et al*., 2010) was used to generate a *glf* (MMCAP1_0814) deletion mutant. The YCpMmyc1.1 genome contains the

pMycYACTn shuttle vector (Benders *et al.*, 2010), allowing the stable propagation of the genome in yeast and selection for tetracycline resistance (*tet*M) as well as screening for blue colonies via expression of *lac*Z in the mycoplasma.

The tandem repeat coupled with endonuclease cleavage (TREC) constructs to delete *glf* were generated by PCR (Noskov *et al.*, 2010). The CORE component of the construct, containing the endonuclease I-*Sce*I cleavage site, the I-*Sce*I endonuclease (under the gal1 promoter) and URA3 were amplified from YCpMmyc1.1-DtypeIIIres::URA3 (Lartigue *et al.*, 2009) using primers glf CORE-F and glf CORE-R (Table S2). For the PCR, a mixture of DreamTaq Polymerase and Pfu proofreading polymerase (Thermo Scientific, Cat nos. EP0701 and EP0501, respectively) was prepared (1:2.5 vol : vol) in DreamTaq buffer, with an annealing temperature of 52°C and extension time of 2 min and 20 s. The Tandem Repeat (TR) part of the deletion construct was amplified from gDNA isolated from *Mmc* strain GM12 using primers glf TR-F and glf TR-R (Table S2) with the PCR conditions used to amplify the CORE component (see above). The samples were loaded on a low-melting agarose gel, and gel slices were excised and kept at –20°C. To fuse the two PCR products (CORE and TR), the gel slices were heated to 65°C, and about 100 ng of each PCR product was added directly to a standard PCR reaction mix containing the primers glf CORE-F and glf TR-R (Table S2). The PCR conditions were: initial denaturation of 2 min at 94°C, followed by five cycles of 94°C for 1 min, 45°C for 3 min and 72°C for 4 min during which the complementary ends of the CORE and TR fragments acted as primers to join the sequences. This was immediately followed by 35 cycles of 94°C for 45 s, 60°C at 45 s and 72°C for 3 min, and a final elongation step of 15 min at 72°C. The fused PCR products were gel extracted (QIAGEN, Germany) and subsequently used to transform the *S. cerevisiae* strain W303a containing the YCpMmyc1.1 genome, using the LiAc method (Gietz *et al.*, 1992).

Insertion of the construct at the desired locus (i.e. deletion of *glf*) was confirmed using primers glf-upstream-seq, glf-downstream-seq (Table S2), located upstream and downstream of gene.

To generate seamless deletions of *glf*, double stranded cleavage was induced by plating on galactose containing medium, followed by 5-FOA (Carl Roth, Germany) counter selection against the presence of the URA3 gene as described elsewhere (Noskov *et al.*, 2010). The seamless deletion was confirmed by sequencing using the up- and downstream primers described above (Table S2). To verify that the YCpMmyc1.1 genome was not altered otherwise, an *Mmc*-specific multiplex PCR was performed using primers MPCR1_F – S8G_MPCR8_R (Table S2) and the QIAGEN Multiplex PCR Kit (QIAGEN, Cat no. 206143).

The completeness of the YCpMmyc1.1-Δ*glf* genome in yeast was finally confirmed by PFGE. Briefly, yeast agarose plugs were prepared using the CHEF Mammalian Genomic DNA Plug Kit (Bio-Rad), following the protocol described before (Lartigue *et al.*, 2009) and subjected to electrophoresis in a 1% pulsed-field agarose gel (Bio-Rad) in 1× TAE, with a contour-clamped homogeneous electric field (CHEF DR III; Bio-Rad). Prior to PFGE, yeast chromosomal DNA was removed from agarose plugs by treating the agarose plugs with a cocktail of restriction enzymes (AsiSI, FseI and RsrII) that have multiple recognition sites in yeast chromosomes and none in *Mmc* genomes and subsequent electro-removal of fragmented yeast DNA by agarose electrophoresis. After that step, the circular DNA that remained in agarose plugs was BssHII-restricted and finally separated by PFGE. Pulse times were ramped from 60 to 120 s for 24 h at 6V cm^{-1}. After electrophoresis, the gel was stained with SYBR Gold, and PFGE patterns were visualised using a VILBER E-BOX VX5 imager and VisionCapt software.

The intact YCpMmyc1.1-Δ*glf* genome was then transplanted into *Mcc* RE(-) as described before (Lartigue *et al.*, 2009). The resulting mutant strain *Mmc* YCpMmyc1.1-Δ*glf* was confirmed by PCR using the up- and downstream primers, as well as the *Mmc*-specific multiplex PCR (Table S2).

PacBio sequencing and genome assembly

High molecular weight DNA from YCpMmyc1.1-Δ*glf* was sheared in a Covaris g-TUBE (Covaris, Woburn, MA, USA) to obtain 20 kb fragments. After shearing the DNA size distribution was checked on a Fragment Analyzer (Advanced Analytical Technologies, Ames, IA, USA). Six micrograms of the sheared DNA was used to prepare a SMRTbell library with the PacBio SMRTbell Template Prep Kit 1 (Pacific Biosciences, Menlo Park, CA, USA) according to the manufacturer's recommendations. The 1.6 μg of the resulting library was size selected on a BluePippin system (Sage Science, Beverly, MA, USA) for molecules larger than 10 kb. The recovered library was sequenced on one SMRT cell with P6/C4 chemistry and MagBeads on a PacBio RSII system (Pacific Biosciences, Menlo Park, CA, USA) at 240 min movie length. Sequencing yielded 62 211 post filter reads corresponding to 863 Mb with a mean read length of 13 873 bases. Fifty per cent of bases were in reads longer than 18 050 bases.

For the genome assembly, the PacBio module 'RS_HGAP_Assembly.2' in SMRTpipe version v2.3.0 was used. The seed read length cutoff (reads that after error correction and trimming are used for Celera assembler) was set at 21 771 bp. The assembly yielded a contig of 1.104 Mb.

Testing the functionality of the mutated UDP-glucose-4-epimerase using an E. coli*-based complementation assay*

Wild type and mutant *galE* were synthesised using *E. coli* codon optimisation (GenScript) and cloned in the *E. coli* expression vector pEXT20 (Dykxhoorn *et al.*, 1996) using EcoRI and HindIII sites. The synthesised codon optimised *galE* nucleotide sequences can be found in Table S1.

LPS was extracted from *E. coli* whole cells as described above. Purified LPS was analysed in 12% SDS-PAGE gels and western blot or silver staining (Fig. S5). For western blot analysis, a monospecific polyclonal rabbit anti-O7 antigen (Statens Serum Institut, Denmark) and an IRDye 800CW goat anti-rabbit (LI-COR Biosciences) were used as primary and secondary antibodies respectively. Western blots were visualised using an Odyssey CLx Imager (LI-COR Biosciences). LPS silver staining of SDS-PAGE gels was carried out as previously described (Tsai and Frasch, 1982).

Isolation of carbohydrates

Mycoplasma were cultured in 3 l volumes of PPLO media supplemented with 10% horse serum, 0.5% glucose, 0.03% penicillin G, 20 mg ml^{-1} thallium acetate and 0.9 g l^{-1} yeast extract until mid-log phase. Total polysaccharides (CPS and/or EPS) were isolated using slight modification to the protocols published before (Rurangirwa *et al*., 1987; Waite and March, 2002). The pH of the culture was adjusted to pH 5 using glacial acetic acid (Sigma-Aldrich, Cat. no. 100063). The solution was boiled for 1 h, cooled and filtered through a Whatman paper (Schleicher & Schuell, 3MM CHR Cat. no. 3030 931). Two volumes of absolute ethanol were added, and the mixture was stored at 4°C overnight. The precipitate was collected by centrifugation at 2,600 × *g* for 15 min, resuspended in 200 ml of distilled water and stirred at room temperature for 2 h. After a centrifugation step at 5,000 × *g* for 30 min, an equal volume of aqueous phenol was added to the supernatant, and the mixture was incubated at 68°C for 1 h. Following overnight incubation at 4°C, the mixture was again centrifuged at 5,000 × *g* for 30 min, and the phenol extraction was repeated on the aqueous phase. The aqueous phase was transferred to a dialysis bag with a molecular weight cut off 6–8 kDa (Carl Roth, Cat No E666-1) and dialysed against 2 l H_2O for 48 h (H_2O was exchanged eight times), and the dialysed material was precipitated using two volumes of absolute ethanol, kept at 4°C overnight and collected by centrifugation at 1,000 × *g* for 30 min. The pellet was resuspended in 10 ml distilled H_2O and 10 µl of DNAseI (2,000 units ml^{-1}, Pharmacia, Cat. No. 27-0514-01) and 100 µg of RNAse (Sigma Cat.no. R-4875) was added and incubated at 37°C overnight. SDS was added to a final concentration of 0.5%, followed by addition of 100 µg of proteinase K (BRL Life Technologies, Cat. no. 25530015), and the mixture was incubated at 45°C for 24 h. The mixture was transferred to 5 ml of phase lock gel in a 50 ml Falcon tube, one volume of phenol chloroform was added and mixed thoroughly. After centrifugation at 5,000 × *g* for 15 min, the aqueous phase was transferred to a new tube, and the extraction was repeated with phenol chloroform isoamylalcohol (25:24:1) and the aqueous phase was dialysed against 2 l of distilled water for 4 days (using the same dialysis bags as reported above, water was exchanged several times daily). Finally, the carbohydrates were precipitated using 1/10 volume of 3M sodium acetate and four volumes absolute ethanol, centrifuged at 5,000 × *g* for 30 min, and the pellet was dissolved in distilled water, followed by a centrifugation at 21 000 × *g* for 3 min. The supernatant was retained and kept at –20°C until further experiments. The concentration of carbohydrates was estimated using the phenol–sulphuric acid method (Masuko *et al*., 2005).

SDS-PAGE with Coomassie, PAS staining and western blot

Twenty micrograms of isolated carbohydrate were analysed on standard 12% polyacrylamide gels, either stained by Coomassie or PAS, or transferred to nitrocellulose membranes for western blot analysis. Gels were fixed in Methanol/Acetic acid/H_2O (4/1/5) overnight at room temperature, then equilibrated in 7% cold acetic acid at 4°C for 1 h, then stained in 5 gel volumes of 1% periodic acid in 7% cold acetic acid also for 1 h at 4°C. The gel was subsequently washed with 7% acetic acid at 4°C for 24 h, and the color was developed using cold Schiff reagent (Sigma, Cat. No. 84655) at 4°C for 1 h.

For western and dot blot analysis, the method described by Synaptic Systems (Standard protocol for western blots with alkaline phosphatase staining) (http://www.sysy.com/protocols/blot.php) was used. Membranes were blocked for 1 h with PBS-T containing 5% skim milk, then incubated for 1 h with either AMMY10 mAb or rabbit pAb raised against full-length recombinant *Mmm* elongation factor Tu (Jores *et al*., 2009) (produced by BioGenes GmbH, Germany), diluted 1:200 or 1:10, respectively, in blocking solution. After five washes (10 min each in PBS-T), the membranes were incubated with alkaline phosphatase conjugated α-mouse IgM (1:30 000 dilution in blocking solution, Sigma, Cat. No A9688) or alkaline phosphatase conjugated α-rabbit IgG (dilution 1:5,000, Sigma, Cat. no. A2556) for 1 h, again washed five times for 10 min in PBS-T. Afterwards, the membranes were equilibrated with substrate buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$), developed in BCIP/NBT staining solution, and the reaction was stopped by washing the membrane in water three times.

Electron microscopy

Cells were fixed in 150 mM HEPES, pH 7.35, containing 1.5% formaldehyde and 1.5% glutaraldehyde. Fixation was done 30 min at RT and then over night at 4°C. Fixed cell pellets were immobilised in 4% agar, dehydrated in methanol and embedded in Lowicryl HM20 (Polysciences, Germany). After polymerisation by UV-light, ultrathin sections of 25 nm were immunolabelled at RT as follows: primary antibody, AMMY10, at 1:100 in PBS containing 0.05% Tween-20 and 5% skim milk powder (blocking buffer) for 3 h; 3 × 5 min PBS containing 0.05% Tween-20 (washing buffer); secondary antibody (goat anti-mouse IgM coupled to 10 nm gold) 1: 50 in blocking buffer for 1 h; 3 × 5 min in washing buffer; 2 × 2 s in distilled water. Sections were air dried and then stained 5 min in 4% uranyl acetate.

Membrane integrity assay

The test was performed as recently described (Paradis-Bleau *et al*., 2014) with slight modifications regarding the media used. GM12, YCpMmyc1.1 and YCpMmyc1.1-Δ*glf* were plated on PPLO agar plates containing either 20 µg ml^{-1} 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, Thermo Scientific, Cat. no. R0941) or 20 µg ml^{-1} Chlorophenol Red β-D-galactoside (CPRG, Sigma, Cat no. 59767). Color development was evaluated after 72 h of incubation at 37°C.

Determination of the generation time

The strains GM12, YCpMmyc1.1 and YCpMmyc1.1-Δ*glf* were grown in standard Mycoplasma broth medium (Mycoplasma Experience, UK), or on Mycoplasma solid medium containing 0.9% agarose, at 37°C. To determine the generation time in liquid medium, aliquots were taken every 40 min, diluted by

successive 10-fold serial dilution steps and spread on solid medium. The cfus were determined by counting colonies after 4 days of incubation at 37°C with 5% CO_2. End-exponential phase cultures were taken at pH 7.5 when cfu reached 2×10^9 cfu ml^{-1}.

Adhesion to small ruminant cells assay

Adhesion assays were performed using primary lamb cells from carpal joint synovial tissue (LSM 192) cultivated in 24-well tissue culture plates at 37°C and 5% CO_2 to a density of 6×10^4 cells per well using tissue culture medium (MEM Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, 2.5 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Zimmermann *et al.*, 2010).

Mycoplasma strains were grown in 20 ml of standard Mycoplasma culture medium (Mycoplasma Experience, UK) containing 2 μCi (74 kBq) of [U-^{14}C] palmitic acid for 3 days at 37°C with 5% CO_2. Mycoplasma cultures were harvested by centrifugation at 9,000 × *g* for 5 min and were washed three times in buffer A (0.05 M Tris-HCl, pH 7.2, 0.1 M NaCl and 1 mM $CaCl_2$). Mycoplasma cells were re-suspended in buffer A and adjusted to a concentration of 10^9 cfu ml^{-1}.

To determine the saturation of adhesion, 100 μl aliquots of labelled *Mmc* suspensions at 10^4 cfu ml^{-1} to 10^9 cfu ml^{-1} in buffer A were added to each well and incubated for 2 h at 37°C. After removing excess liquid, cells were washed twice with 200 μl of buffer A and subsequently solubilised with 50 μl of 1% (w/v) SDS solution in buffer A for 2 h at 37°C with shaking. The lysis suspension of each well was then transferred into a vial, air dried and complemented with 3 ml of scintillation cocktail containing 5 g l^{-1} PPO (2,5-Diphenyloxazol from Merck and Toluol from Fluka). Counts per min were measured in a Scintillation spectrometer (Perkin-Elmer Liquid Scintillation AnalyzerTri-Carp 2910 TR). Results are given as mean values of three independent assays with the corresponding standard errors. Aliquots of labelled mycoplasma suspension were measured to determine the incorporation of [U-^{14}C] palmitic acid per cfu *Mmc*.

The Kruskal–Wallis one-way analysis of variance by ranks was performed using Prism 6 for MAC OS X Version 6.0e (GraphPad Software, Inc, La Jolla, CA, USA), as this is a non-parametric test that can compare more than one sample.

Serum sensitivity assay

Mmc strains GM12, YCpMmyc1.1 and YCpMmyc1.1-Δ*glf* were cultivated in PPLO media containing 5% horse serum at 37°C overnight and aliquoted before snap freezing and storage at –80°C. The concentration (cfu ml^{-1}) was determined. Serum sensitivity was tested as previously described (Lees-Miller *et al.*, 2013) with minor modifications to adapt for mycoplasma. Fifty microliters of serial dilutions of mycoplasma was mixed with 50 μl of fresh caprine serum derived from a healthy goats (IACUC ref. no. 2014.26) and incubated at 37°C for 30 min. The added fresh serum was either heat-inactivated at 56°C for 30 min or neat. Only fresh serum, at most 3 days old, was used as we observed that storage of serum at 4°C had a negative effect on the read out of the assay, i.e. loss of killing activity. Five microliters of each mixture was spotted in duplicate onto PPLO agar plates and incubated at 37°C for up to 3 days.

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Supporting information

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