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New insights into *Mycoplasma mycoides* subspecies *mycoides* Small Colony secreted exopolysaccharides

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INTRODUCTION

Mycoplasma mycoides subsp. *mycoides* Small Colony (MmmSC) is the causal agent of contagious bovine pleuropneumonia (CBPP), a severe contagious infection of cattle. This disease is responsible for major losses in African livestock and the European 90's re-emerging outbreaks demonstrated that CBPP was still a threat for developed countries. MmmSC belongs to the class of *Mollicutes*, bacteria that are notably characterized by the lack of a cell wall and a small genome size.

Little is known about MmmSC virulence mechanisms but MmmSC exopolysaccharides (EPS) is a good virulence factor candidate. Previous works showed that MmmSC produced a galactose polymer linked to a lipid moiety : the galactan (Buttery & Plackett, 1960). Galactan is reported to form a thick polysaccharide layer surrounding MmmSC cells, often referred to as a pseudo-capsule. The immunogenicity of galactan, isolated from cells pellet, has been demonstrated (Shiffrine & Gourlay, 1965). The same results were observed with free EPS extracted from culture supernatants (Hudson, 1967) leading to the hypothesis that free EPS and bound galactan could be identical. However free EPS stayed unidentified as it was difficult to distinguish it from culture medium constituents. Besides, MmmSC cultures on solid medium revealed a characteristic phenotype mixture of translucent (TR) and opaque (OP) colonies. This phenotype variation is related to an ON/OFF switch of the glucose phosphotransferase system permease gene that can be revealed with a specific monoclanal antibody "3F3" (Gaurivaud, 2004) (**Fig. 5**). These phenotypes could be linked with the presence or absence of capsular material.

To study the production of MmmSC free EPS *in vitro*, we have designed culture and polysaccharides extractions conditions that enhance the final yield and its purity. The objective was allow a subsequent composition and structural analysis. We have also performed an electron-microscopic examination of MmmSC colonies stained with ruthenium red to identify the capsule constituents and try to unravel the link between capsule production and free EPS secretion . The final aim was to propose a schematic pathway for the MmmSC EPS production.

MATERIAL AND METHODS

Two MmmSC strains were used in this work:
- PG1, the reference strain. Its genome is completely sequenced and annotated.
- Afadé, an African pathogenic strain already used in virulence studies .

OP and TR colonies can be identified with these two strains and their phenotypes are stable.

Free EPS extraction method

MmmSC culture in PPLO medium (37°C, 5%CO2)

Mycoplasmas washed once in PBS

Incubation in Synthetic Medium Supplemented or not with 3g/L of glucose or other carbone source (37°C , 5%CO2)

-Enumeration (CFU/mL)
-pH

Supernatant + Trichloroacetic acid 10% (2h, 4°C)

Supernatant + 9 volumes of cold acetone (24h, -20°C)

Free EPS extract → **Monosaccharide composition (IEHPLC) and structure (NMR) analysis**

-carbohydrate content determined by Dubois method
- SDS-Page stained with PAS
-Immunodetection with bovine convalescent serum

Variants studies

Variants isolation

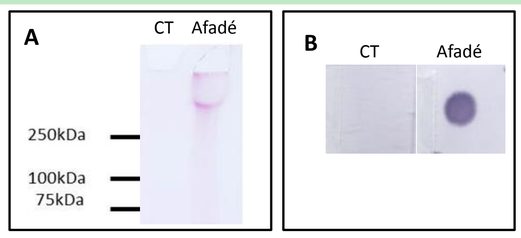
Colony blotting with mAb 3F3

OP (3F3 OFF) TR (3F3 ON)

Electron microscopy
Free EPS extraction method

RESULTS

Validation of the free EPS extraction method

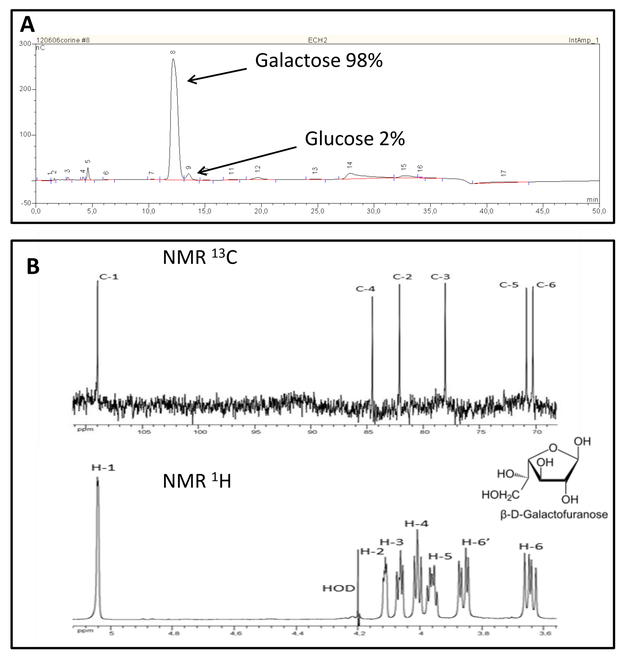


Preliminary analysis of MmmSC Afadé free EPS after using culture conditions in synthetic medium and extraction protocol on culture supernatants.

Polyacrylamide gel electrophoresis of enriched free EPS fraction of MmmSC Afadé stained with PAS **(A)**.
Immunodetection with bovine convalescent serum of enriched free EPS fraction of Mmm Afadé spotted on a nitrocellulose membrane **(B)**.
CT corresponds to the synthetic medium used in the assays.

Fig. 1 -MmmSC Afadé produces free EPS which is recognized by bovine convalescent serum
-No medium contaminations

Composition and structure of the free EPS

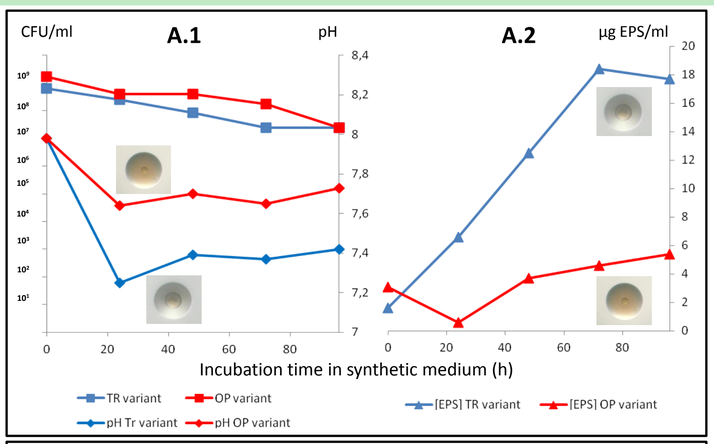


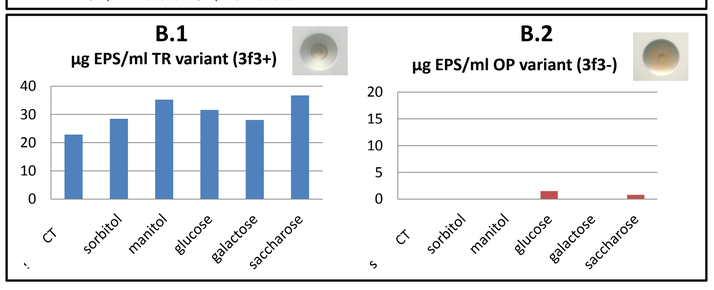
Analysis of MmmSC Afadé free EPS

Liquid chromatogram (IEHPLC) of the monosaccharides extract content **(A)** NMR ^{13}C and NMR ^{1}H of the free EPS extract **(B)**

Fig. 2 Free EPS is a β(1-6) D-galactofuranose composed of galactose and glucose

Variants studies : free EPS production



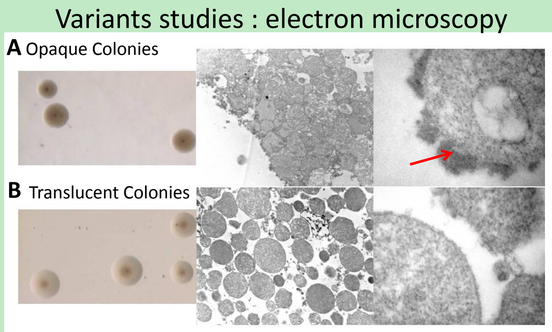


Comparison of survival, culture pH and free EPS production with the two isolated MmmSC Afadé variants

A. Survival expressed in colony forming units (CFU/ml) and pH in synthetic medium **(A.1)** and carbohydrate assays on the free EPS extrated from supernantants in µg/ml **(A.2)** at different time of sampling in hours for the translucide (TR) and opaque (OP) variants.
B. Carbohydrate content in µg/ml of the free EPS extracted from supernantants after 72h of incubation in synthetic medium supplemented with 3g of various sugar sources for the TR **(B.1)** and the OP **(B.2)** variants.
CT corresponds to the manipulation in a non supplemented synthetic medium.

Fig. 4 -The medium is less acidified by the opaque variant
-The opaque variant produces very little free EPS as compared to the translucent variant

Variants studies : electron microscopy



Electron micrograph of MmmSC PG1 cells variants. Surrounding capsular material is present (A) or absent (B)

Fig. 3 -The opaque variant is capsulated
-The translucent variant is not

Variants studies : PtsG expression

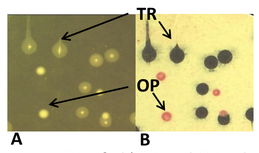


Figure 5. **MmmSC is a mixture of translucent (TR) and opaque (OP) colonies due to a switch ON/OFF of the PtsG gene.**

MmmSC Afadé TR and OP colonies on agar PPLO plates **(A)** Colonies immunoblotting with mAb 3F3 and red Ponceau staining **(B)**

Fig. 5 The opaque variant is not recognized by "3F3" PtsG monoclonal antibody

CONCLUSIONS

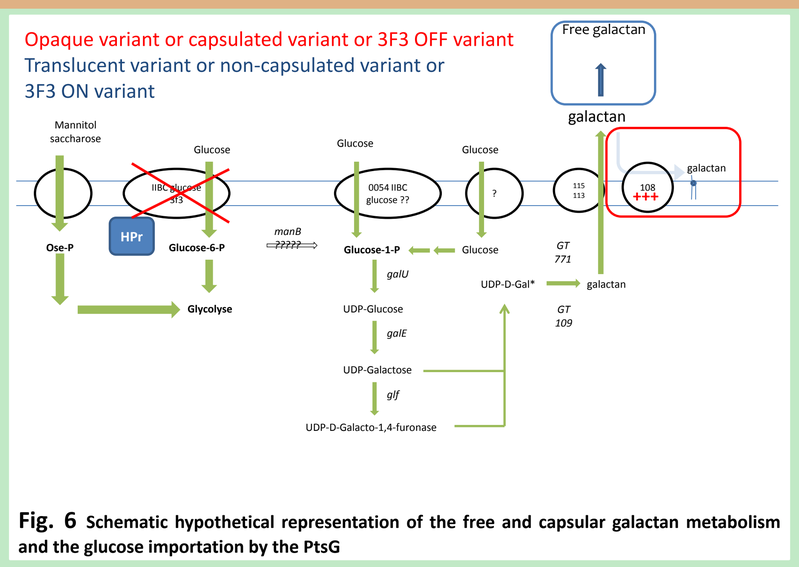


Fig. 6 **Schematic hypothetical representation of the free and capsular galactan metabolism and the glucose importation by the PtsG**

In this work, we have designed culture conditions and a supernatant polysaccharidic extraction protocol that prevent growth medium contaminations **(Fig. 1A)** and allow the extraction of a polysaccharidic material which is immunogenic **(Fig. 1B)**. MmmSC free EPS contains the same monosaccharide composition as cell-bound galactan : 98% of galactose and 2% of glucose **(Fig. 2A)** and D-galactofuranose linked in β(1-6) base structure **(Fig. 2B)**. MmmSC has been described as a pseudo-capsulated organism for more than 50 years. When it is cultivated on PPLO agar plates, MmmSC appears to be a mixture of translucent and opaque colonies. Electron microscopy experiments performed on the two isolated variants has shown that the opaque variant presents capsular material at its surface **(Fig. 3A)**. Translucent variant does not **(Fig. 3B)**. Similar observations were made in other bacteria species like *Vibrio*.

Interestingly, the capsulated variant free EPS production is minimal by comparison to the non-capsulated variant **(Fig. 4A2)**, whatever the glucose addition or the carbon source tested **(Fig. 4B)**. Protein MSC_108, annotated as a glycosyltransferase, is significantly over-expressed by the capsulated variant (unpublished results) and a TMHMM *in silico* analysis indicates a transmembrane localization for this protein. Therefore this protein could be implicated in the attachment of the galactan on the opaque variant cell surface. We have also observed that the medium is less acidified by this variant **(Fig. 4A1)**. It could be due to a lack of glucose 6-P importation in glycolysis pathway because of the absence of the PtsG **(Fig. 6)**. All these elements show that EPS production seems to be independent of glycolysis **(Fig. 6).** These results and hypothesis pave the way for future research on MmmSC EPS metabolism and its possible role in virulence.

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