

Functional and antigenic properties of GlpO from *Mycoplasma mycoides* subsp. *mycoides* SC: characterization of a flavin adenine dinucleotide-binding site deletion mutant

Daniela F. BISCHOF, Edy M. VILEI*, Joachim FREY

Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Länggass-Strasse 122,
P.O. Box, CH-3001 Bern, Switzerland

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Abstract – L- α -glycerophosphate oxidase (GlpO) plays a central role in virulence of *Mycoplasma mycoides* subsp. *mycoides* SC, a severe bacterial pathogen causing contagious bovine pleuropneumonia (CBPP). It is involved in production and translocation of toxic H₂O₂ into the host cell, causing inflammation and cell death. The binding site on GlpO for the cofactor flavin adenine dinucleotide (FAD) has been identified as Gly₁₂–Gly₁₃–Gly₁₄–Ile₁₅–Ile₁₆–Gly₁₇. Recombinant GlpO lacking these six amino acids (GlpO Δ FAD) was unable to bind FAD and was also devoid of glycerophosphate oxidase activity, in contrast to non-modified recombinant GlpO that binds FAD and is enzymatically active. Polyclonal monospecific antibodies directed against GlpO Δ FAD, similarly to anti-GlpO antibodies, neutralised H₂O₂ production of *M. mycoides* subsp. *mycoides* SC grown in the presence of glycerol, as well as cytotoxicity towards embryonic calf nasal epithelial (ECaNEp) cells. The FAD-binding site of GlpO is therefore suggested as a valuable target site for the future construction of deletion mutants to yield attenuated live vaccines of *M. mycoides* subsp. *mycoides* SC necessary to efficiently combat CBPP.

CBPP / L- α -glycerophosphate oxidase / FAD-binding site / targeted deletion mutant / neutralizing antibody

1. INTRODUCTION

Glycerol metabolism is described to play a central role in virulence of *Mycoplasma mycoides* subsp. *mycoides* SC, the etiological agent of contagious bovine pleuropneumonia (CBPP) [4, 13, 14, 20], as well as in *Mycoplasma pneumoniae* [9]. Glycerol at physiological concentrations is incorporated and phosphorylated to obtain α -glycerol-3-phosphate (G3P) by *M. mycoides* subsp. *mycoides* SC via the highly active ATP-dependent glycerol transporter system GtsABC and then further metabolised to dihy-

droxyacetone phosphate (DHAP) in the presence of oxygen by the L- α -glycerophosphate oxidase (GlpO, also named α -glycerol-3-phosphate oxidase) with the release of the highly toxic compound hydrogen peroxide H₂O₂ [20]. Scattered electron microscopy and Triton X-114 partitioning revealed GlpO to be membrane-located in *M. mycoides* subsp. *mycoides* SC with a significant part of the protein being surface-exposed [13], which explains the fact that the mycoplasma is able to release significant amounts of H₂O₂ into the growth medium.

Using a cell culture infection model for *M. mycoides* subsp. *mycoides* SC it was shown that H₂O₂ is not only released by the mycoplasma

* Corresponding author: edy.vilei@vbi.unibe.ch

but directly translocated into the eukaryotic host cells causing cytotoxicity and cell death [13]. *M. mycoides* subsp. *mycoides* SC strains generally possess the glycerol ABC transporter GtsABC for the assimilation of glycerol with the exception of strains from recent European outbreaks (period 1980–1999) producing significantly lower amounts of H₂O₂ [8, 20] and showing a very low cytotoxicity level towards eukaryotic host cells in the presence of glycerol compared to most other strains [4, 13]. Blocking the L- α -glycerophosphate oxidase GlpO by monospecific anti-GlpO antibodies hampers the production and release of H₂O₂ and concomitantly inhibits cytotoxicity of *M. mycoides* subsp. *mycoides* SC towards embryonic calf nasal epithelial (ECaNEp) cells [13]. These results indicate that GlpO may act as a useful antigen target to induce protective immunity against CBPP.

Furthermore, since GlpO-promoted H₂O₂ production is a central virulence attribute of *M. mycoides* subsp. *mycoides* SC, the *glpO* gene represents an ideal target for mutations in order to better attenuate vaccine strains, whose residual virulence is of concern. In this respect, it has to be mentioned that the live vaccine strains T1/44 and T1/Sm are currently used to vaccinate against CBPP [18]. However, both vaccines have been shown to contain a fully active glycerol transport system GtsABC and an enzymatically active L- α -glycerophosphate oxidase GlpO, which results in the release of significant amounts of H₂O₂ in the presence of physiological concentrations of glycerol [4, 21] and cytotoxicity against ECaNEp cells [4]. This feature might be the cause of unwanted side reactions encountered with these vaccines [17] and of CBPP elicitation in cattle upon inoculation of T1-derived vaccines via the endobronchial route [11].

In the current work, we studied the basic, structural and functional features of GlpO of *M. mycoides* subsp. *mycoides* SC with the aim of identifying mutation sites in the *glpO* gene for future live vaccine development. In this view, the mutations should result in (i) loss of H₂O₂-producing enzymatic activity; (ii) attenuation of currently used vaccines; and (iii) preservation of the antigenic properties of

GlpO required to elicit neutralising antibodies like those induced by native GlpO [13]. GlpO (EC 1.1.3.21) is an enzyme that belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of the donor with oxygen as the acceptor. It employs one cofactor, flavin adenine dinucleotide (FAD). In biochemistry, FAD is a redox cofactor involved in several important metabolic reactions. FAD can exist in two different redox states and its biochemical role usually involves alternating between these two states. FAD can be reduced to FADH₂, whereby it accepts two hydrogen atoms. FADH₂ is an energy-carrying molecule, and the reduced coenzyme can be utilised as a substrate for oxidative phosphorylation. FADH₂ is re-oxidised to FAD, which makes it possible to produce two moles of the universal energy carrier ATP. The ADP portion of FAD interacts mostly via residues from the first β -strand- α -helix- β -strand ($\beta\alpha\beta$) fold in the N-terminal portion of GlpO, which includes the Gly-X-Gly-X-X-Gly fingerprint that interacts with the diphosphate moiety of FAD [7].

The present study investigated the function of recombinant GlpO from *M. mycoides* subsp. *mycoides* SC, determining the FAD-binding site and the potential of mutant GlpO Δ FAD, i.e., the recombinant GlpO without the FAD-binding site Gly₁₂-Gly₁₃-Gly₁₄-Ile₁₅-Ile₁₆-Gly₁₇, to induce antibodies neutralising cytotoxicity of *M. mycoides* subsp. *mycoides* SC.

2. MATERIALS AND METHODS

2.1. Strains, growth conditions and DNA extraction

M. mycoides subsp. *mycoides* SC strain Afadé, a highly virulent field strain isolated in 1968 in Cameroon, was used for the virulence studies unless otherwise stated. Mycoplasmal cultures were grown in a standard mycoplasma medium (Axcell Biotechnologies, St. Genis-L'Argentière, France) at 37 °C to a density of approximately 5×10^8 cells per mL. For genetic manipulation and subcloning, *Escherichia coli* strains DH5 α and BL21(DE3) were used. The pETHIS-1 expression vector [15] was used for expression of recombinant N- and C-terminal polyhistidine fusion proteins.

2.2. ECaNEp cells

ECaNEp cells were prepared from foetuses obtained from a local abattoir and were maintained in minimal essential medium (MEM)-Earle medium supplemented with 7% foetal calf serum and penicillin (100 IU/mL) in 24-well microtiter plates and used at a confluent density of 2×10^5 cells per well, at 37 °C in a humidified 5% CO₂ atmosphere [13]. Foetal calf serum and cell culture media were purchased from Seromed (Biochrom, Munich, Germany). The cells were routinely screened for contamination by mycoplasmas using PCR or for contamination by bovine viral diarrhoea virus using immunostaining [16].

2.3. Bioinformatics

Comparison of the GlpO protein sequence of strain Afadé with the EMBL/GenBank database was performed using the BLAST program blastp [1]. The PSIPRED Protein Structure Prediction Server v2.6 [12] was used to predict the secondary structure of GlpO of strain Afadé.

Since no 3D structures are annotated in the 3D databases for GlpO of *M. mycoides* subsp. *mycoides* SC, a search for 3D domains for bacterial GlpO sequences within available sequences from the NCBI site was performed. Among the 14 GlpO structures found, L- α -glycerophosphate oxidase 2RGH:A from *Streptococcus* [7] was selected for comparison with the GlpO of *M. mycoides* subsp. *mycoides* SC. The 3D-Mol Viewer software from the Vector NTI Advance 10.3.0 package (Invitrogen AG, Basel, Switzerland) was used to display the 3D protein structure model of 2RGH:A with its FAD molecule. The SWISS-MODEL software [2], accessible via the ExPASy web server, was used to display GlpO of *M. mycoides* subsp. *mycoides* SC taking 2RGH:A from *Streptococcus* as the template.

2.4. Cloning and expression of recombinant proteins

The *glpO* gene of *M. mycoides* subsp. *mycoides* strain Afadé was first amplified with the primers Afadé_EcoRI and MmmSC_GlpO_BamHI (Tab. I) containing the restriction sites for *EcoRI* and *BamHI*, respectively. As a template for PCR amplification, plasmid pETHIS-1::glpO containing mutagenised TGG₇₁₀ codons in the *glpO* gene [13] was used. The pETHIS-1 vector was amplified with the primers pETHIS-1_EcoRI and pETHIS-1_BamHI (Tab. I) to

give the linearised vector pETHIS-1m harbouring *EcoRI* and *BamHI* restriction sites. This procedure was used to create GlpO fusion with a polyhistidine tail at the C-terminus only. Purified recombinant GlpO carrying the 10xHis-tagged C-terminus was shown to be enzymatically more active under the in vitro conditions used in this study compared to GlpO with polyhistidine tags at both N-terminal and C-terminal ends.

The codons for the putative FAD-binding site Gly₁₂-Gly₁₃-Gly₁₄-Ile₁₅-Ile₁₆-Gly₁₇ on *glpO* were removed using the overlap extension-PCR method [5] to obtain the *glpO* Δ FAD gene. The codons for the putative FAD-binding site located at nucleotides (nt) 34–51 downstream of the ATG start codon of the *glpO* sequence of strain Afadé with EMBL/GenBank accession number AJ581566 were removed subsequently with (i) primer pair MmmSC_glpO_FAD1F and MmmSC_glpO_FAD1R (Tab. I) to remove bases 34–39 coding for Gly₁₂-Gly₁₃, followed by (ii) primer pair MmmSC_glpO_FAD2F and MmmSC_glpO_FAD2R (Tab. I) to remove bases 40–45 coding for Gly₁₄-Ile₁₅, and (iii) primer pair MmmSC_glpO_FAD3F and MmmSC_glpO_FAD3R (Tab. I) to remove bases 46–51 coding for Ile₁₆-Gly₁₇. The products were cloned into pETHIS-1m by flanking *EcoRI* and *BamHI* cleavage sites. The constructs were analysed by DNA sequencing and the final clone was introduced into *E. coli* BL21(DE3) for expression. The expression of the cloned *glpO* Δ FAD gene was induced by the addition of 1 mM IPTG at mid-exponential phase and further incubation for 4 h at 30 °C. Following induction, the polyhistidine-tailed fusion protein GlpO Δ FAD was purified via Ni²⁺ chelation chromatography on a Ni-NTA column under denaturing conditions as described previously for other proteins [15, 19]. The fractions were dialysed and analysed on SDS-10% polyacrylamide gels.

Immunoblot analysis with mouse anti-His antibody (GE Healthcare, Otelfingen, Switzerland) (at a dilution of 1:3000) was carried out by standard methods [3], using SDS-10% polyacrylamide gels for separation of the proteins, which were blotted onto nitrocellulose membranes. Phosphatase-labelled conjugate goat anti-mouse immunoglobulin G (heavy plus light chains) (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) at a dilution of 1:2000 was used for the visualisation of recombinant, polyhistidine-tailed proteins on blots.

2.5. In vitro activity assay

Both recombinant, polyhistidine-tailed proteins GlpO and GlpO Δ FAD were also purified under

Table 1. Oligonucleotide primers.

Primer	Sequence (5'-3') ^a
Afadé_EcoRI	GATCgaattcATGAAGCAAACAAAAGTTGATATTG
MmmSC_GlpO_BamHI	ATATggatccTTTCCATGGAAGAATAGCTTC
MmmSC_glpO_FAD1F	GATATTTGTATCATTGGAATTATTGGTGCTTCTGTTGCAAGAGAA
MmmSC_glpO_FAD1R	AGCACCAATAATTCCAATGATACAAATCAACTTTTGTGTTGCTT
MmmSC_glpO_FAD2F	GATATTTGTATCATTATTGGTGCTTCTGTTGCAAGAGAACTAGCA
MmmSC_glpO_FAD2R	AACAGAAGCACCAATAATGATACAAATCAACTTTTGTGTTGCTT
MmmSC_glpO_FAD3F	GATATTTGTATCATTGCTTCTGTTGCAAGAGAACTAGCAAATTT
MmmSC_glpO_FAD3R	TCTTGCAACAGAAGCAATGATACAAATCAACTTTTGTGTTGCTT
pETHIS-1_EcoRI	GATCgaattcTCTCCTTCTTAAAGTTAAACAAAA
pETHIS-1_BamHI	ATATggatccCACCATCACCATCACCATC

^a Lower-case letters indicate nucleotides added to create restriction enzyme recognition sites for cloning.

native conditions in order to maintain their biological activity. For this purpose, *E. coli* BL21(DE3) cells expressing the recombinant protein GlpO were suspended in buffer A (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) followed by sonification in a Branson Sonifier and binding for 1 h at 4 °C to Ni-NTA beads. The beads were then washed with buffer C (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with buffer D (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). Recombinant GlpOΔFAD was purified by suspending the corresponding IPTG-induced *E. coli* cells in buffer B (50 mM NaH₂PO₄, 300 mM NaCl, 1 mM imidazole, pH 6.5) followed by sonification in a Branson Sonifier and binding for 1 h at 4 °C to Ni-NTA beads. The beads were then washed with buffer B and eluted with buffer D. The fractions were analysed on SDS-10% polyacrylamide gels.

The activities of purified recombinant proteins GlpO and GlpOΔFAD were measured by their ability to produce H₂O₂ in the presence of glycerol. The activity assay was performed in the presence of filter-sterilised incubation buffer (67.7 mM HEPES, 140 mM NaCl, 7 mM MgCl₂, pH 7.3) supplemented with 9 mM ATP, 65 nM FAD and 6 mM α-glycerol-3-phosphate (G3P), to which recombinant GlpO or recombinant GlpOΔFAD was added at a final 20 µg/mL concentration. The production of H₂O₂ was measured with the peroxide test (Merck KgaA, Darmstadt, Germany) as described previously [20].

2.6. Fluorescence measurements

Fluorescence excitation and emission spectra of the proteins was measured with a Perkin-Elmer LS50 dual wavelength fluorescence spectrophotome-

ter. The emission spectrum (370–600 nm) at an excitation wavelength of 350 nm and the excitation spectrum (250–410 nm) at an emission wavelength of 430 nm were recorded as described previously [6].

2.7. Sera, polyclonal antibodies and immunoglobulin purification

Immune sera directed against recombinant GlpO or GlpOΔFAD were obtained by subcutaneous immunisation of rabbits with 80 µg of purified recombinant polyhistidine-tailed proteins in 500 µL of phosphate-buffered saline (PBS) buffer (50 mM Na₂HPO₄/NaH₂PO₄, 140 mM NaCl, pH 8.0), mixed with 500 µL of Adjuvant 10 (Gerbu Biotechnik GmbH, Gaiberg, Germany). Primary immunisation was followed by booster injections with 80 µg of the respective protein 2 and 4 weeks later. The rabbits were bled 21 days after the last booster immunisation. Antisera were prepared from the blood samples and stored at -20 °C.

Immunoglobulin G (IgG) fractions from decomplemented rabbit anti-GlpO and anti-GlpOΔFAD sera were purified with the HiTrap Protein G kit (Amersham Pharmacia Biotech, Uppsala, Sweden) as directed by the manufacturer. Protein concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Witec AG, Littau, Switzerland).

2.8. Quantification of H₂O₂ and inhibition assay

To measure H₂O₂ production, strains of *M. mycoides* subsp. *mycoides* SC were grown in mycoplasma culture medium for 3 days at 37 °C to a density of approximately 5 × 10⁸ cells/mL. The culture was centrifuged at 8 000 × g for 10 min at 4 °C, washed

once in incubation buffer, resuspended in pre-warmed incubation buffer at 37 °C at a density of 10⁹ cells/mL, portioned in aliquots of 1 mL, and incubated at 37 °C for 1 h. To induce H₂O₂ production, glycerol was added to the mycoplasma suspensions at a final concentration of 100 μM, representing the physiological concentration of glycerol in bovine serum. The production of H₂O₂ was measured with the peroxide test (Merck KgaA) as described previously [20] at time zero and 1, 2, 5, 10, 20 and 40 min after the addition of glycerol. In order to measure the inhibitory potential of anti-GlpO or anti-GlpOΔFAD antibodies, mycoplasmas were pre-treated with IgG preparations of either anti-GlpO or anti-GlpOΔFAD antiserum at concentrations of 10 μg/mL for 30 min at 37 °C, followed by two washes with PBS buffer.

2.9. Detection of translocation of H₂O₂ from infecting mycoplasmas into ECaNEp cells

To monitor intracellular H₂O₂ and accompanying reactive oxygen species (ROS) in ECaNEp cells, the formation of dichlorofluorescein derivatives after cleavage of the ester groups of nonfluorescent 5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester (CM-H₂DCFDA; Molecular Probes, Eugene, OR, USA) by H₂O₂-dependent oxidation was measured as already described [13]. The ECaNEp cells were incubated for 1 h with 10 μM CM-H₂DCFDA and washed once with MEM-Earle medium. Cells were then infected with *M. mycoides* subsp. *mycoides* SC strain Afadé at a multiplicity of infection (MOI) of 500 mycoplasmas per cell in the presence or absence of 100 μM glycerol. To assess blocking of the GlpO activity, *M. mycoides* subsp. *mycoides* SC cells were pre-treated with anti-GlpO or anti-GlpOΔFAD IgG at 8 to 28 μg/mL. Intracellular H₂O₂ was monitored 20 min after infection with mycoplasmas by fluorescence microscopy using a Nikon Eclipse TE 300 microscope. Note that all steps involving CM-H₂DCFDA, including handling of this chemical, were performed in the dark.

3. RESULTS

3.1. FAD-binding site Gly-X-Gly-X-X-Gly of GlpO

The results obtained with the BLAST program blastp showed that the 387-aa GlpO

of *M. mycoides* subsp. *mycoides* SC strain Afadé (EMBL/GenBank accession number CAE46342) has strong homologies to several bacterial glycerol-3-phosphate oxidases and glycerol-3-phosphate dehydrogenases. In some cases, these similar proteins are larger than 450 aa in length and the GlpO-homologous portions reside in their N-termini (not shown).

The secondary structure prediction of GlpO of strain Afadé indicates the presence of a βαβ fold in its N-terminal portion, which includes the Gly-Gly-Gly-Ile-Ile-Gly fingerprint that is assumed to interact with the diphosphate moiety of FAD (Fig. 1). A comparison with protein 2RGH:A, the L-α-glycerophosphate oxidase from *Streptococcus* [7], demonstrated the presence of two putative G3P-binding sites also in GlpO from *M. mycoides* subsp. *mycoides* SC. They are located in the region spanning aa residues 310–350, close to the C-terminus (Fig. 1).

Protein 2RGH:A, whose 3D structure is known, was chosen as a model to envisage the possible 3D structure of GlpO from *M. mycoides* subsp. *mycoides* SC. 2RGH:A showed the three Gly residues in the Gly-X-Gly-X-X-Gly fingerprint (in this case Gly₃₉-Gly₄₀-Gly₄₁-Ile₄₂-Thr₄₃-Gly₄₄) in a βαβ fold in the vicinity of the diphosphate moiety of FAD (Fig. 2A), which corresponds to the putative FAD-binding site Gly₁₂-Gly₁₃-Gly₁₄-Ile₁₅-Ile₁₆-Gly₁₇ in the GlpO of *M. mycoides* subsp. *mycoides* SC strain Afadé (Fig. 2B).

3.2. Structural properties and enzymatic activity of recombinant GlpO and GlpOΔFAD

Immunoblot analysis of recombinant proteins GlpO and GlpOΔFAD, both carrying a 10xHis-tagged C-terminus, with anti-His antibodies revealed two protein bands of 45 and 90 kDa for GlpO and one single protein band of 44 kDa for GlpOΔFAD (Fig. 3A). Both polyclonal monospecific anti-recombinant GlpO and GlpOΔFAD sera strongly reacted with a 43-kDa protein and poorly reacted with 85- and 125-kDa proteins from total antigen of *M. mycoides* subsp. *mycoides* SC strain Afadé (Fig. 3B).

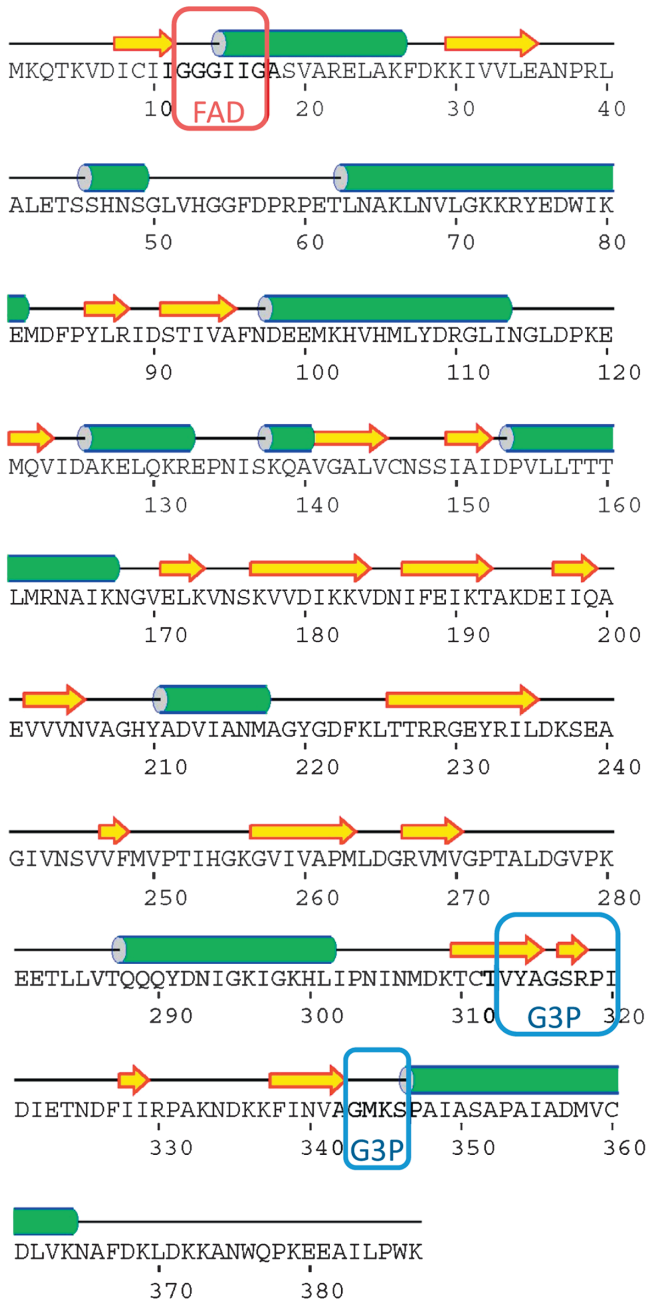


Figure 1. Secondary structure of GlpO of *M. mycoides* subsp. *mycoides* SC strain Afadé. Boxes represent FAD-binding (red) and putative G3P-binding (blue) segments, as defined previously [7]. Yellow arrows and green cylinders represent β -sheets and α -helices, respectively, as obtained by the protein structure prediction program PSIPRED. (For a colour version of this figure, please consult www.vetres.org.)

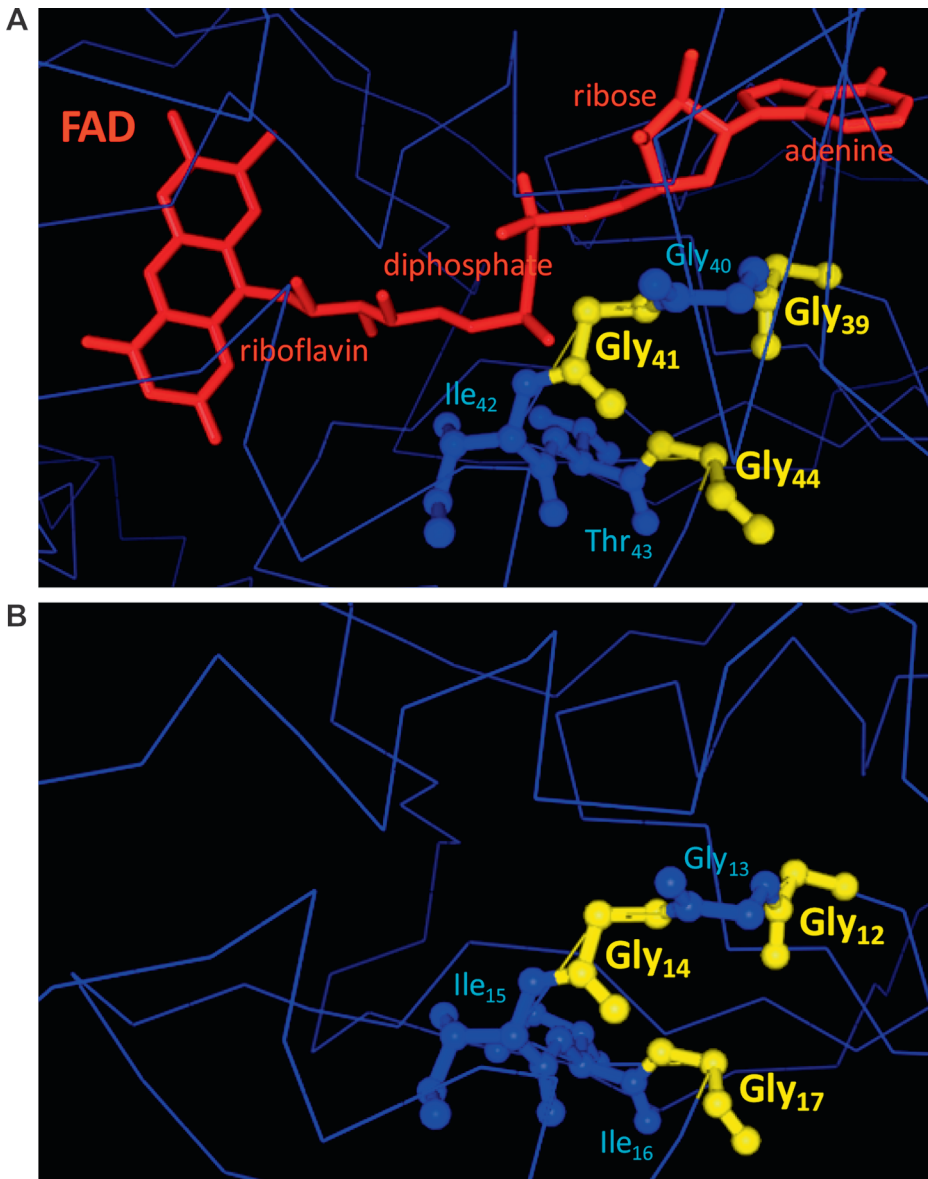


Figure 2. Three-dimensional model showing the FAD-binding site of GlpO. The “ball and stick” views were obtained with the 3D-Mol Viewer program to display: (A) the predicted structure model of the FAD-binding site of 2RGH:A of *Streptococcus* [7] and its bound FAD molecule (“stick” view); and (B) the SWISS-MODEL-simulated structure of the FAD-binding site of GlpO of *M. mycoides* subsp. *mycoides* SC strain Afadé obtained by taking 2RGH:A from *Streptococcus* as the template (FAD is not displayed by SWISS-MODEL-generated structure simulation). The protein chains (excluding the FAD-binding sites Gly-X-Gly-X-X-Gly) are illustrated as blue wires. The displayed FAD molecule is shown in red and the amino acids X of the FAD-binding sites are shown in blue while their three amino acids Gly are displayed in yellow. (For a colour version of this figure, please consult www.vetres.org.)

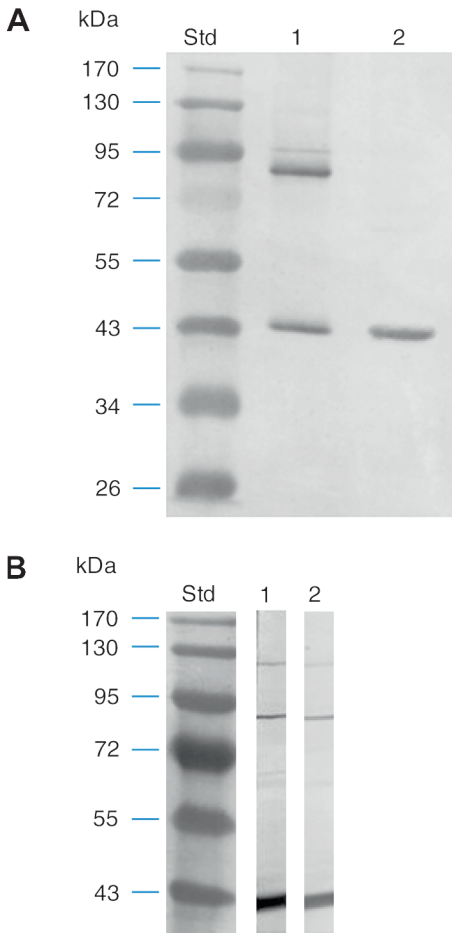


Figure 3. Immunoblot detection of GlpO and GlpO Δ FAD. (A) One-microgram samples of purified recombinant GlpO (lane 1) and GlpO Δ FAD (lane 2) were run by SDS-10% polyacrylamide gels, transferred onto a nitrocellulose membrane and probed with anti-His antibody. (B) Total antigen (approximately 10 μ g per lane) of *M. mycoides* subsp. *mycoides* SC strain Afadé was separated on SDS-10% polyacrylamide gels and immunoblotted using either anti-GlpO (lane 1) or anti-GlpO Δ FAD antiserum (lane 2) at a dilution of 1:1000. Std, molecular mass standards.

To test the activity of both enzymes, recombinant proteins GlpO and GlpO Δ FAD were purified under native conditions and tested for their functional and enzymatic activities. A final concentration of 20 μ g/mL for both recombinant

proteins was used in the assays. Under in vitro conditions, recombinant GlpO of *M. mycoides* subsp. *mycoides* SC oxidised G3P with release of 2 μ g/mL H₂O₂ into the assay solution after 10 min incubation and 10 μ g/mL H₂O₂ after 40 min incubation. In contrast, recombinant GlpO Δ FAD showed no release of H₂O₂ into the assay solution.

3.3. Spectroscopic analysis of GlpO and GlpO Δ FAD

It was observed that cell lysates of GlpO-producing *E. coli* had a yellowish appearance, whereas cell lysates with the protein GlpO Δ FAD had a whitish appearance, like *E. coli* BL21(DE3) harbouring vector pETHIS-1 without insert. This indicates that cell lysates with recombinant GlpO contained protein-bound flavin (i.e. bound FAD), whereas GlpO Δ FAD did not bind FAD.

In order to confirm this, fluorescence spectra of the two recombinant proteins GlpO and GlpO Δ FAD were measured and compared with the spectra of an FAD solution, the latter displaying an emission maximum at 525 nm. The fluorescence spectra of recombinant GlpO-samples displayed the typical protein-derived excitation maximum at 280 nm and also showed an emission maximum at 525 nm (Fig. 4), indicating that recombinant GlpO contained bound FAD that must have been acquired during overexpression in *E. coli*. In contrast, fluorescent spectroscopic analysis of recombinant GlpO Δ FAD displayed solely an excitation maximum at 280 nm and no peak visible at 525 nm in the emission spectrum, showing that this recombinant protein did not contain bound FAD (Fig. 4). These results indicate that recombinant GlpO Δ FAD lacking the putative FAD-binding site Gly₁₂-Gly₁₃-Gly₁₄-Ile₁₅-Ile₁₆-Gly₁₇ was unable to acquire and bind the cofactor FAD when expressed in *E. coli*, hence explaining why it is enzymatically inactive.

3.4. Neutralising activity of anti-GlpO Δ FAD antibodies

With respect to future vaccine design, it appears highly desirable to use enzymatically

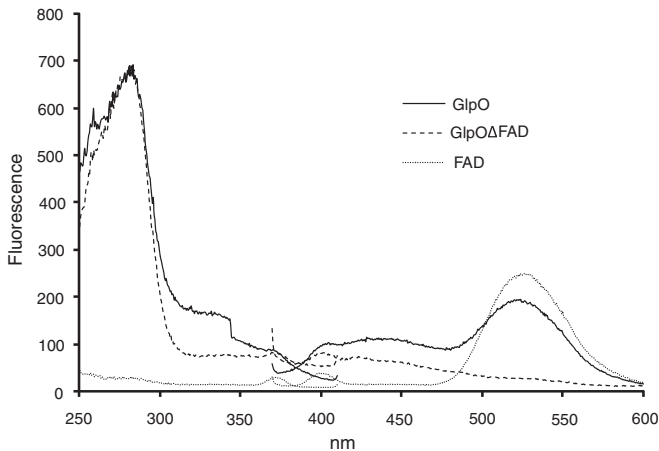


Figure 4. Fluorescence excitation and emission spectra of GlpO, GlpO Δ FAD and FAD. Spectra of GlpO (4.6 μ M), GlpO Δ FAD (1.2 μ M) and FAD (7.7 μ M) in 20 mM PBS were recorded. Excitation wavelength was 350 nm, and emission wavelength was 430 nm. Excitation spectra were measured at 250–410 nm; emission spectra were measured at 370–600 nm. The bold line represents the recombinant protein GlpO, the dashed line represents GlpO Δ FAD and the dotted line represents free FAD.

inactive GlpO eliciting antibodies in immunised animals able, like those directed against native GlpO [13], to neutralise GlpO and thereby obliterating the cytotoxic effect of *M. mycoides* subsp. *mycoides* SC. The neutralising activity of rabbit antibodies that were induced by immunisation with recombinant GlpO Δ FAD was therefore assessed.

As observed with rabbit anti-GlpO [13], de-complemented rabbit anti-GlpO Δ FAD serum and protein G-purified IgG prepared thereof were shown to specifically block the release of H₂O₂ by *M. mycoides* subsp. *mycoides* SC strain Afadé grown in liquid medium supplemented with 100 μ M glycerol. Pre-immune serum or IgG of the unvaccinated rabbit had no inhibitory effect. Consequently, the GlpO Δ FAD protein lacking the FAD-binding site and the native GlpO have similar capacities to induce GlpO-neutralising antibodies.

In order to evaluate whether anti-GlpO Δ FAD antibodies are also able to neutralise translocation of H₂O₂ and the accompanying oxygen radicals from *M. mycoides* subsp. *mycoides* SC into ECaNEp cells, and the resulting cytotoxic effects, ECaNEp cells were pre-treated with CM-H₂DCFDA to detect intracellular

oxidation of this compound by fluorescence microscopy. Infection with the African *M. mycoides* subsp. *mycoides* SC field strain Afadé produced a strong induction of fluorescence in ECaNEp cells 20 min after the addition of glycerol, reflecting the presence of intracellular H₂O₂, an event followed by cell death. No fluorescence was detected in the control experiment where ECaNEp cells were infected with the Afadé strain without the addition of glycerol (Fig. 5). Intracellular oxidation of CM-H₂DCFDA in ECaNEp cells as visualised by fluorescence was inhibited when infecting *M. mycoides* subsp. *mycoides* SC were pre-treated with antibodies directed either against GlpO (Fig. 5C) or against GlpO Δ FAD (Fig. 5D). This showed that GlpO Δ FAD is able to induce antibodies that prevent H₂O₂ translocation from infecting *M. mycoides* subsp. *mycoides* SC to the host cells.

4. DISCUSSION

With the aim of designing new live vaccines against CBPP using specific, targeted, attenuating mutations in the genome of *M. mycoides* subsp. *mycoides* SC, we investigated the role of the

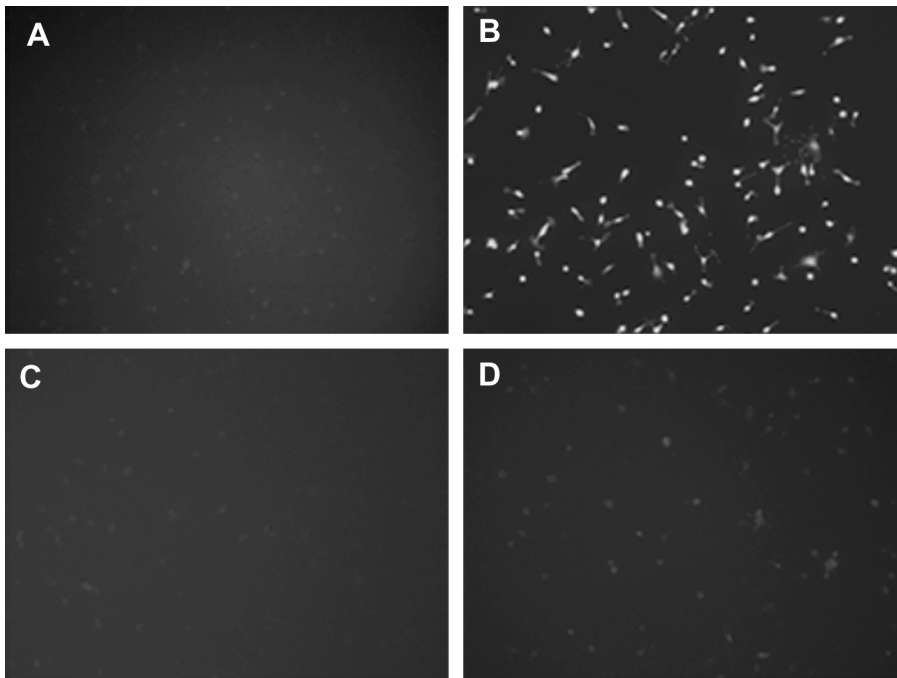


Figure 5. Fluorescence micrographs showing detection of intracellular H_2O_2 in ECaNEp cells infected with *M. mycoides* subsp. *mycoides* SC at an MOI of 500 mycoplasmas per cell. ECaNEp cells 20 min after infection with strain Afadé in the absence (A) and in the presence of glycerol (B), or with strain Afadé pre-treated with anti-GlpO IgG (C) or with anti-GlpO Δ FAD IgG (D) in the presence of glycerol.

FAD-binding site of the L- α -glycerophosphate oxidase GlpO, a central enzyme for the virulence of this pathogen [4, 13, 14].

Spectroscopic analysis revealed that the modified recombinant GlpO Δ FAD – the GlpO protein-mutant whose FAD-binding site was knocked out by recombinant gene technology – was devoid of bound FAD while the intact recombinant GlpO did contain bound FAD. It is interesting to note at this point that purified recombinant GlpO, in contrast to GlpO Δ FAD, showed two protein bands on SDS-polyacrylamide gels and immunoblots, the larger band being attributed to the formation of a dimeric aggregate of GlpO, in accordance with the finding that this oxidase is a dimeric protein in other organisms [6, 7]. The observation that GlpO Δ FAD is present uniquely as a monomer on immunoblots suggests that the lack of the FAD-binding site (or the failure of binding

FAD) also changed structural properties of this protein. Furthermore, the FAD-binding site of GlpO was demonstrated to play a central role in the enzymatic activity of the oxidase, as shown by the inability of GlpO Δ FAD to oxidise α -glycerol-3-phosphate (G3P) and to produce toxic H_2O_2 .

Antibodies that were raised in rabbits against purified recombinant GlpO Δ FAD, like anti-GlpO antibodies, were able to (i) recognise three bands (representing monomer, dimer and a possible trimer on immunoblots) of GlpO from total proteins of *M. mycoides* subsp. *mycoides* SC; (ii) block the H_2O_2 production of *M. mycoides* subsp. *mycoides* SC metabolising glycerol; and (iii) neutralise the resulting cytotoxicity towards host cells in an in vitro assay.

Taken together, these results suggest that the removal of the FAD-binding site from the *glpO* gene of *M. mycoides* subsp. *mycoides* SC

provides an ideal means to attenuate cytotoxicity of future live vaccines, with preserved capacity to elicit efficient GlpO-neutralising antibodies in the host. In order to replace the *glpO* wild type gene in the chromosome of *M. mycoides* subsp. *mycoides* SC with the *glpO* Δ FAD allele for creation of a targeted deletion mutant, novel, more powerful methods for allelic replacement still need to be developed since this organism does not seem to be particularly apt to homologous recombination [10].

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REFERENCES

- [1] Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J., Basic local alignment search tool, *J. Mol. Biol.* (1990) 215:403–410.
- [2] Arnold K., Bordoli L., Kopp J., Schwede T., The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling, *Bioinformatics* (2006) 22:195–201.
- [3] Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., Struhl K., Current protocols in molecular biology, John Wiley & Sons, Inc., New York, 1999.
- [4] Bischof D.F., Janis C., Vilei E.M., Bertoni G., Frey J., Cytotoxicity of *Mycoplasma mycoides* subsp. *mycoides* small colony type to bovine epithelial cells, *Infect. Immun.* (2008) 76:263–269.
- [5] Braman J., Papworth C., Greener A., Site-directed mutagenesis using double-stranded plasmid DNA templates, *Methods Mol. Biol.* (1996) 57:31–44.
- [6] Claiborne A., Studies on the structure and mechanism of *Streptococcus faecium* L-alpha-glycerophosphate oxidase, *J. Biol. Chem.* (1986) 261:14398–14407.
- [7] Colussi T., Parsonage D., Boles W., Matsuoka T., Mallett T.C., Karplus P.A., Claiborne A., Structure of α -glycerophosphate oxidase from *Streptococcus* sp.: a template for the mitochondrial α -glycerophosphate dehydrogenase, *Biochemistry* (2008) 47:965–977.
- [8] Djordjevic S.P., Vilei E.M., Frey J., Characterization of a chromosomal region of *Mycoplasma* sp. bovine group 7 strain PG50 encoding a glycerol transport locus (*gtsABC*), *Microbiology* (2003) 149: 195–204.
- [9] Hames C., Halbedel S., Hoppert M., Frey J., Stülke J., Glycerol metabolism is important for cytotoxicity of *Mycoplasma pneumoniae*, *J. Bacteriol.* (2009) 191:747–753.
- [10] Janis C., Bischof D., Gourgues G., Frey J., Blanchard A., Sirand-Pugnet P., Unmarked insertional mutagenesis in the bovine pathogen *Mycoplasma mycoides* subsp. *mycoides* SC: characterization of a *lppQ* mutant, *Microbiology* (2008) 154:2427–2436.
- [11] Mbulu R.S., Tjipura-Zaire G., Lelli R., Frey J., Pilo P., Vilei E.M., et al., Contagious bovine pleuropneumonia (CBPP) caused by vaccine strain T1/44 of *Mycoplasma mycoides* subsp. *mycoides* SC, *Vet. Microbiol.* (2004) 98:229–234.
- [12] McGuffin L.J., Bryson K., Jones D.T., The PSIPRED protein structure prediction server, *Bioinformatics* (2000) 16:404–405.
- [13] Pilo P., Vilei E.M., Peterhans E., Bonvin-Klotz L., Stoffel M.H., Dobbelaere D., Frey J., A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp. *mycoides* Small Colony, *J. Bacteriol.* (2005) 187:6824–6831.
- [14] Pilo P., Frey J., Vilei E.M., Molecular mechanisms of pathogenicity of *Mycoplasma mycoides* subsp. *mycoides* SC, *Vet. J.* (2007) 174:513–521.
- [15] Schaller A., Kuhn R., Kuhnert P., Nicolet J., Anderson T.J., MacInnes J.I., et al., Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*, *Microbiology* (1999) 145:2105–2116.
- [16] Schweizer M., Peterhans E., Oxidative stress in cells infected with bovine viral diarrhoea virus: a crucial step in the induction of apoptosis, *J. Gen. Virol.* (1999) 80:1147–1155.
- [17] Thiaucourt F., Yaya A., Wesonga H., Huebschle O.J.B., Tulasne J.J., Provost A., Contagious bovine pleuropneumonia: a reassessment of the efficacy of vaccines used in Africa, *Ann. N. Y. Acad. Sci.* (2000) 916:71–80.
- [18] Thiaucourt F., Aboubakar Y., Wesonga H., Manso-Silvan L., Blanchard A., Contagious bovine pleuropneumonia vaccines and control strategies: recent data, *Dev. Biol. (Basel)* (2004) 119:99–111.

[19] Vilei E.M., Abdo E.-M., Nicolet J., Botelho A., Gonçalves R., Frey J., Genomic and antigenic differences between the European and African/Australian clusters of *Mycoplasma mycoides* subsp. *mycoides* SC, *Microbiology* (2000) 146:477–486.

[20] Vilei E.M., Frey J., Genetic and biochemical characterization of glycerol uptake in *Mycoplasma mycoides* subsp. *mycoides* SC: its impact on H₂O₂

production and virulence, *Clin. Diagn. Lab. Immunol.* (2001) 8:85–92.

[21] Vilei E.M., Correia I., Ferronha M.H., Bischof D.F., Frey J., β -D-Glucoside utilization by *Mycoplasma mycoides* subsp. *mycoides* SC: possible involvement in the control of cytotoxicity towards bovine lung cells, *BMC Microbiol.* (2007) 7:31.