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The surface-localised α-enolase of *Mycoplasma suis* is an adhesion protein

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ABSTRACT

Mycoplasma suis belongs to the haemotrophic mycoplasmas which colonise red blood cells of a wide range of vertebrates. Adhesion to red blood cells is the crucial step in the unique lifecycle of *M. suis*. Due to the lack of a cultivation system, identification of adhesion structures has been difficult. So far, only one adhesion protein, i.e. MSG1 was identified. In order to determine further adhesion molecules of *M. suis*, we screened genomic *M. suis* libraries and performed Southern blot hybridisation analyses of genomic *M. suis* DNA. The α-enolase of *M. suis* was identified and analysed genetically and functionally. The encoding gene has 1623 bp in size. The deduced amino acid sequence showed an overall identity of 59.6–65.1% to α-enolases of other pathogenic mycoplasmas. The 540 aa *M. suis* α-enolase displays a size extension of about 90 aa in comparison to α-enolases of other mycoplasmas. Recombinant α-enolase expressed in *Escherichia coli* demonstrated immunogenicity in experimentally infected pigs. Immunoblot, confocal laser scanning microscopy and immune electron microscopy analysis using antibodies against recombinant α-enolase, indicate the membrane and surface localisation of native α-enolase in *M. suis*, though no typical signal sequences exist. Furthermore, we showed that recombinant α-enolase binds to porcine erythrocyte lysate in a dose-dependent manner. *E. coli* transformants which express α-enolase on their surface acquire the ability to adhere to porcine red blood cells. In conclusion, our observations indicate that α-enolase could be involved in the adhesion of *M. suis* to porcine red blood cells.

1. Introduction

Mycoplasma (M.) suis, a member of the uncultivable haemotrophic mycoplasmas could be found as surface parasites on or intracellularly in porcine RBCs (Hoelzle, 2008; Groebel et al., 2009). *M. suis* infections are distributed worldwide and cause the pig industry serious economic losses (Hoelzle, 2007; Ritzmann et al., 2009). Current studies revealed that *M. suis* could also infect humans after close contact to *M. suis*-infected animals (Yang et al., 2000; Yuan et al., 2009). In these reports symptoms like mild pyrexia, haemolytic anemia, and icterus are described indicating a zoonotic potential.

The adhesion of *M. suis* is crucial for its unique RBC lifestyle. Adhesion is mediated by fine fibrils that bridge a narrow space between *M. suis* and the RBC (Hoelzle, 2008; Zachary and Basgall, 1985). However, adhesion mediating proteins and underlying adhesion mechanisms are unknown so far. Due to the lack of culture systems only one *M. suis* adhesion protein, i.e. MSG1, has been proven so far. MSG1 is a surface-localised multitasking protein with enzymatic function in the carbohydrate metabolism (Hoelzle et al., 2007d). For other mycoplasmas, e.g. *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, it is known that cell

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adhesion obviously is a complex and multifactorial process which requires multiple proteins (Burgos et al., 2006; Chaudhry et al., 2007). Therefore, it is assumed that *M. suis* possesses further adhesion proteins or adhesion-associated proteins. Recently, other immunoreactive proteins of *M. suis* have been identified including α-enolase (Hoelzle et al., 2007c). The glycolytic α-enolase could be involved in the interaction of *M. suis* with its host cell, since α-enolase of streptococci and *Aeromonas hydrophila* is known to perform an alternative task as a surface protein which binds to plasminogen or fibronectin and thus mediates cell adhesion and invasion (Esgleas et al., 2008; Pancholi and Fischetti, 1997, 1998; Sha et al., 2009). Furthermore, the α-enolase of *Mycoplasma fermetans* is surface-localised and binds to plasminogen, indicating an important role in cell adherence and invasion (Yavlovich et al., 2004, 2007). In the present study, we identified the α-enolase gene of *M. suis* and recombinantly expressed the corresponding protein in *Escherichia coli* for functional characterisation. We describe the *M. suis* α-enolase as an immunogenic and surface-localised protein that could act as an adhesion factor in *M. suis*.

2. Materials and methods

2.1. Plasmids, experimental pig sera and M. suis isolates

Escherichia (E.) coli K12 strains Top10 and LMG194 (Invitrogen, Basel, Switzerland) were used to clone and express the library clones and the *M. suis* α-enolase gene. For DNA manipulation and protein expression, the following vectors were used: pUC19 (Roche-Diagnostics, Rotkreuz, Switzerland), pGFP (green fluorescent protein; Clontech, Allschwil, Switzerland), and the arabinose-inducible pBad-MycHisA (Invitrogen). Blood sera from experimentally infected pigs and *M. suis* isolates were available from previous studies (Hoelzle et al., 2009; Ritzmann et al., 2009). All animal experiment were performed according to the law and approved by an ethical committee.

2.2. Purification of M. suis and M. suis membranes

M. suis was purified from the blood of experimentally infected animals. Blood was drawn at maximum bacteraemia as confirmed by microscopic examination of acridine orange-stained blood smears and by *M. suis*-specific quantitative real-time Light Cycler-PCR (Hoelzle et al., 2007b). Whole blood was collected in Alsever's solution (Sigma, Buchs, Switzerland) as anticoagulant. Purification of whole *M. suis* cells was performed as described previously (Hoelzle et al., 2006). *M. suis* membranes were purified by osmotic lysis (Razin, 1983). The resulting cytoplasmic and membrane proteins were both resuspended in 500 μl of Beta buffer (0.15 M NaCl, 0.01 M β-mercaptoethanol (Sigma), 0.05 M Tris–HCl (pH 7.4) and stored at −80 °C.

2.3. Library construction and sequence analysis

Genomic *M. suis* DNA was purified as described elsewhere (Hoelzle et al., 2003a). Library construction was performed by MWG Biotech (Ebersberg, Germany). Briefly, *M. suis* DNA fragments averaging about 1.5 kb (Ms_library1) and 2.5–3.0 kb (Ms_library2) were ligated into a blunt-end cut, *Sma*I-digested pUC19 vector and transformed into *E. coli* Top10.

2.4. Identification of M. suis α-enolase

For the identification of the *M. suis* α-enolase gene the digoxigenin (DIG)-dUTP labelled *Mycoplasma penetrans* α-enolase gene (GenBank: BA000026) was used as a probe for colony hybridisation of the *M. suis* library clones. The library clone SG1-66 reacting with the *M. penetrans* α-enolase probe was sequenced. The containing DNA insert with the highest identity to the *M. penetrans* α-enolase gene was used to produce a *M. suis*-specific *α-enolase* DIG-labelled probe for Southern blot hybridisation of *Hind*III-digested genomic *M. suis* DNA (Hoelzle et al., 2003a). The detected 2.1 kb DNA fragment was cloned into pUC19 vector and sequenced. Nucleotide sequences were analysed using the FastA algorithm (Biocomputing, University Zurich, www.bio.uzh.ch/docu/fasta/). Putative open reading frames (ORFs) were determined using the ORF finder program (www.ncbi.nlm.nih.gov/projects/gorf/). Translation of ORFs to amino acid sequences was performed by taking into account the alternative genetic codon usage of mollicutes (UGA is read as tryptophan codon (UGA_{Trp}) instead of as a stop codon; translation table 4).

2.5. Mutation-combined PCR of the complete M. suis α-enolase gene and cloning

To circumvent the *Mycoplasma*-specific translational barrier to the UGA codon (tryptophan *vs.* stop codon) a mutation-combined PCR method (Hames et al., 2005) was used to convert the TGA triplet within the *M. suis* α-enolase gene into a UGG triplet coding for tryptophan. The resulting PCR fragments were sequenced and checked for the correct replacement of the UGA triplet by UGG. Then, the mutated α-enolase gene was ligated into pBadMycHisA and transformed into *E. coli* LMG194 (*E. coli_eno*). Furthermore, the α-enolase was fused with *gfp* (*E. coli_eno_gfp*; *E. coli_gfp*) as described previously (Hoelzle et al., 2007d).

2.6. Expression and purification of recombinant α-enolase of M. suis (rMsEno)

Expression of recombinant *M. suis* α-enolase was performed as described previously (Hoelzle et al., 2007d). His-tagged proteins were purified by nickel affinity chromatography (Qiagen, Hombrechtikon, Switzerland) from the cytoplasmic (CP) and outer membrane (OM) compartments as described previously (Hoelzle et al., 2003b, 2007d). Expression of GFP was analysed using a Leica Photomicroscope DMI RXA2 (Leica, Mannheim, Germany) using the L5 filter for green fluorescence.

2.7. Production of anti-rMsEno immune serum

A polyclonal mono-specific anti-rMsEno immune serum (RαMsEno) was raised by immunisation of a rabbit

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using 0.5 mg of purified protein and Freund's complete adjuvant or Freund's incomplete adjuvant (Sigma), respectively (Hoelzle et al., 2007d).

2.8. RBC membrane (PECL) purification

Haemoglobin-free RBC membranes were prepared from the blood of healthy pigs by hypotonic lysis as described previously (Hoelzle et al., 2007d). The resulting RBC membranes (designated as porcine erythrocyte lysate, PECL) were resuspended in PBS and stored at −80 °C until used.

2.9. SDS-PAGE, immunoblot, and dot blot

SDS-PAGE and immunoblots were performed according to standard procedures (Laemmli, 1970; Towbin et al., 1979). Immunoblots were probed with *E. coli* absorbed pig and rabbit sera.

Surface localisation of α-enolase in *E. coli* was checked by dot blot. Arabinose-induced and non-induced *E. coli_eno* were dropped onto a nitrocellulose membrane, and incubated with RαMsEno (pre-adsorbed with *E. coli*). The purity of the *E. coli* membrane fractions (immunoblot) as well as the intactness of the *E. coli* cells (dot blot) were tested using a mouse monoclonal antibody to the RNA polymerase beta (RpoB) subunit of *E. coli* (Abcam, Cambridge, UK; 1:1000).

2.10. ELISA assay for α-enolase-binding

An enzyme-linked immunosorbent assay (ELISA) and biotinylated rMsEno was used to analyse the binding of rMsEno to PECL as described elsewhere (Hoelzle et al., 2007c). Inhibition of binding was monitored by pre-incubating biotinylated rMsEno with RαMsEno IgG, respectively (1:100 in dilution buffer). Rabbit pre-immune serum served as negative control.

2.11. Adhesion of recombinant E. coli_eno to RBCs

Attachment of *E. coli_eno* to porcine RBCs was performed using GFP recombinants (*E. coli_eno_gfp* and *E. coli_gfp*). Ethanol fixed porcine blood smears were incubated with bacteria for 30 min at 37 °C. To eliminate unbound bacteria, blood smears were washed five times with PBS. Adhesion was visualised by using a Leica SP 2 confocal microscope, and images were captured by using Image J program and Imaris Software with a Soft Imaging System (SIS) camera, and FIVE Software (Soft Imaging System GmbH, Münster, Germany).

2.12. Immunogold transmission electron microscopy (TEM)

M. suis-infected RBCs were diluted (1:10) in PBS and settled on 12 nm carbon-coated cover slips (1200 × *g*, 5 min) using a Cytospin 2 centrifuge (Shandon, Dako-Diagnostica, Zug, Switzerland). Unreacted aldehydes were blocked with 0.1 M glycine (Carl Roth, Karlsruhe, Germany) in PBS. Non-specific binding was reduced by incubation of samples in 3% PBS-buffered foetal calf serum (FCS, Biochrom, Schaffhausen, Switzerland; 40 min). Then, RBCs were stained with RαMsEno (1:100), followed by gold-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:100; Sigma).

After immunostaining, samples were fixed with 2.5% PBS-buffered glutaraldehyde solution (GA grade I; Sigma), and post-fixed in 1% osmium tetroxide (Fluka Chemie, Buchs, Switzerland). Finally, the cover slips were dehydrated with increasing concentrations (70–100%) of ethanol, embedded in epon/araldite (Fluka Chemie), and sectioned. The grids with ultrathin sections were contrasted with 4% uranyl acetate (Fluka Chemie) and lead citrate as previously described (Groebel et al., 2009). The samples were examined by using a Phillips CM 100 transmission electron microscope.

2.13. Confocal laser scanning microscopy (CLSM)

RBCs of *M. suis*-infected blood (40 μl) were diluted in 2.5 ml PBS containing 10 mM glucose and 0.1% BSA (Sigma). Cells were fixed in 4% PBS-buffered paraformaldehyde (Sigma) containing 0.01% GA (grade I) and seeded onto poly-L-lysine-coated glass slides (SuperFrost; Menzel, Braunschweig, Germany). Unreacted aldehydes were blocked with 0.1 M glycine (Carl Roth) in PBS. Non-specific binding of antibodies was reduced by incubation of samples in 3% FCS in PBS. The RBC surface and *M. suis* cells were stained with mouse anti-pig CD235a (glycophorin A) monoclonal antibody (1:100; Phar-Mingen, BD Biosciences) and RαMsEno serum (1:100), followed by tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-mouse IgG (1:100; Sigma), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100; Sigma), respectively. The staining procedure resulted in TRITC-labelled RBCs (red) and FITC-labelled *M. suis* cells with the surface-exposed α-enolase (green), respectively. Confocal microscopy was performed with a Leica SP 2 confocal microscope.

3. Results

3.1. Identification of M. suis α-enolase

Screening of genomic *M. suis* DNA libraries and *Hind*III digested genomic *M. suis* DNA by Southern blot analysis using DIG-labelled *M. penetrans* and *M. suis* α-enolase probes resulted in the identification of the entire 1623 bp ORF for the *M. suis* α-enolase. The found *M. suis* enolase ORF codes for a 540 aa protein with a predicted molecular mass of 58.7 kDa and an isoelectric point of 5.80. The predicted α-enolase showed the highest identity with α-enolases of *M. penetrans*, *Mycoplasma capricolum*, and *Mycoplasma mycoides* (65.1%, 59.6%, and 58.6%, respectively).

The 540 aa *M. suis* α-enolase displays a size extension of about 90 aa in comparison to α-enolases of other mycoplasmas which range from 450 aa (*M. penetrans*) to 458 aa (*M. genitalium*). The characteristic and highly conserved α-enolase signature motif (aa-sequence VLIKvNQIGTLSET) was identified in *M. suis* α-enolase by using the program PROSITE of the Swiss Institute of

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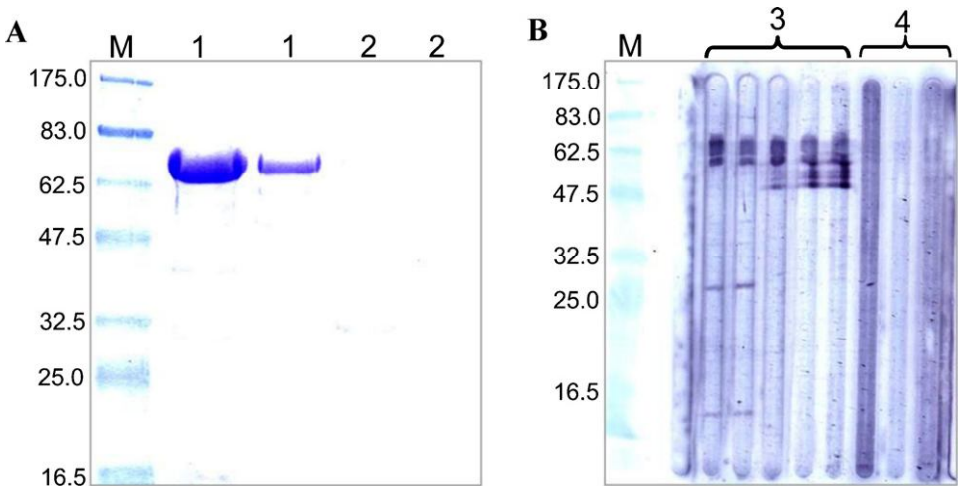

Fig. 1. (A) SDS-PAGE of recombinant *M. suis* α-enolase (rMsEno); (1) purified recombinant protein from arabinose-induced *E. coli_eno* transformants; (2) non-induced control preparation; (B) immunoblot analysis of rMsEno reacting with (3) five sera of *M. suis*-infected pigs and (4) sera of three *M. suis*-negative pigs. M, molecular weight marker (kDa).

Bioinformatics (http://www.expasy.org/tools/scanprosite/scanview-doc.html). The substrate-binding pocket, the cofactor-binding site for Mg^{2+} and the dimer interface region was also found in the *M. suis* α-enolase.

3.2. Expression and characterisation of recombinant M. suis α-enolase in E. coli

In order to circumvent the inability to express *Mycoplasma* proteins in *E. coli* due to the atypical UGA codon usage (translation table 4; UGA is translated as tryptophan instead of stop), a mutation-combined PCR was used to replace the one UGA triplet (aa position 389) by an UGG triplet. The overexpression of the *M. suis* α-enolase (rMsEno) in *E. coli* resulted in a protein migrating on SDS-PAGE with a molecular mass of 63 kDa (Fig. 1A).

The recombinant protein was used to raise an *M. suis* α-enolase-specific rabbit polyclonal antiserum (RαMsEno). The specificity of RαMsEno was demonstrated by immunoblot analysis. RαMsEno specifically reacted with the 63 kDa band of rMsEno, and with a 59 kDa band of *M. suis* whole cell preparation (Fig. 2A + B). The size shift between the rMsEno and the *M. suis* α-enolase is caused by the 6-His tag and the *myc* epitope fused to rMsEno. The specific immunoreactivity of rMsEno was confirmed by immunoblot using five sera of an experimentally *M. suis*-infected piglet, taken during the time course of the disease, and sera of three non-infected piglets as negative control (Fig. 1B). Only the sera of the *M. suis*-infected piglet showed clear reactions with rMsEno.

3.3. Localisation of rMsEno in E. coli

The sub cellular localisation of α-enolase in *E. coli* transformants was analysed by immunoblot analysis using *E. coli* cytoplasm and membrane preparation, and the RαMsEno. As shown in Fig. 2B, a 63 kDa band corresponding to rMsEno could be detected in both purified compartments. The purity of the membrane fraction was demonstrated by immunoblot analysis using a monoclonal antibody against the intracellular RNA polymerase beta subunit (RpoB) of *E. coli*. A reaction was observed with the cytoplasmic fraction, while no reaction was found with the membrane preparation (Fig. 2C). Dot blot analysis with intact recombinant *E. coli* cells provided evidence of surface localisation of rMsEno in *E. coli*. Arabinose-induced *E. coli_eno* cells showed specific reactions with RαMsEno (Fig. 2D) indicating the surface expression of α-enolase in *E. coli* transformants. In contrast, non-induced *E. coli_eno* did not react with RαMsEno. Intactness of the *E. coli* cells was proven using a monoclonal antibody against the intracellular RNA polymerase beta subunit (RpoB) of *E. coli*. No reaction could be observed either with induced or non-induced *E. coli-eno* (data not shown).

3.4. Localisation of α-enolase in M. suis

To localise α-enolase in *M. suis* whole cell, cytoplasmic, and membrane protein preparations were analysed. The hyper immune serum specifically reacted with the 59 kDa α-enolase in all three preparations, reinforcing the evidence of cytoplasmic and membrane-localised *M. suis* α-enolase (Fig. 2A). The surface exposure of *M. suis* α-enolase was demonstrated by TEM analysis of immunogold stained *M. suis*-infected porcine RBCs. Gold-labelled *M. suis* α-enolase was observed on the surface of *M. suis* cells near the attachment site to the RBC (Fig. 3). In addition, surface accessibility of *M. suis* α-enolase was further proven by CLSM (Fig. 4).

3.5. Alpha-enolase is involved in attachment to porcine RBCs

The binding activity of rMsEno to PECL was tested by ELISA. Microtiter plates were coated with decreasing concentrations of purified PECL proteins and incubated with biotinylated rMsEno. Purified rMsEno bound *in vitro* to PECL in a dose-dependent manner. The rMsEno binding to the lysate was specifically blocked by pre-incubation of

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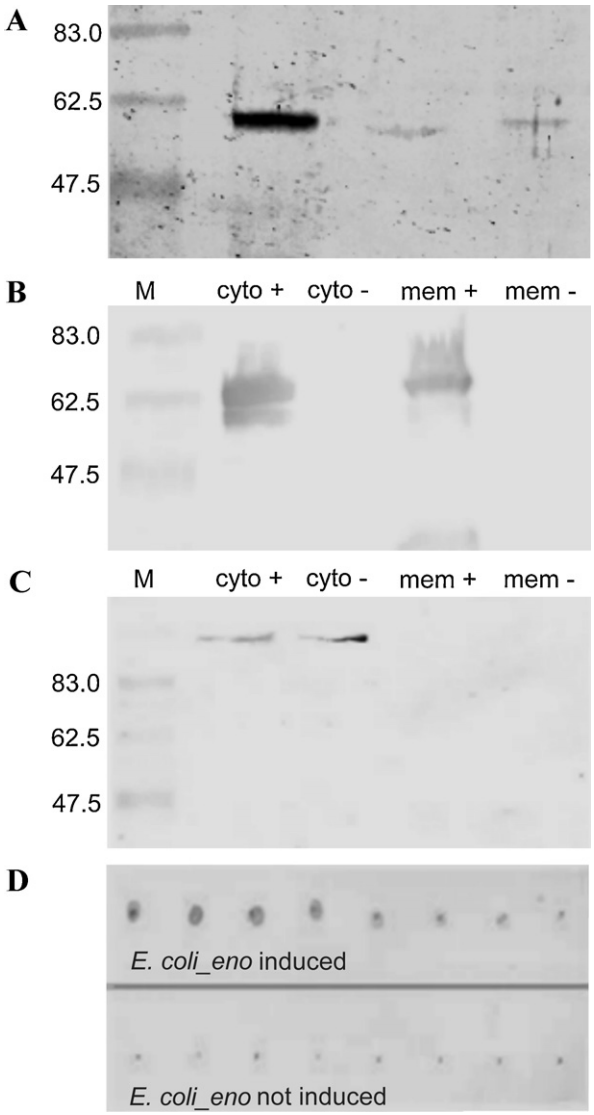

Fig. 2. Determination of the sub cellular localisation of α-enolase in *M. suis* and in *E. coli* using immunoblot analysis (A) *M. suis* α-enolase was found in all three *M. suis* fractions (*Ms*: *M. suis* whole cell; cyto: cytoplasm; and mem: membrane); (B) rMsEno could be detected in the cytoplasma (cyto+) and membrane fraction (mem+) of arabinose-induced *E. coli* transformants. Purifications from non-induced *E. coli* transformants (cyto-, mem-) showed no reaction. (C) Purity of the membrane fractions was confirmed by using a monoclonal antibody against *E. coli* RpoB. The *E. coli* cytoplasmic fractions (cyto+, cyto–) reacted with the antibody, the *E. coli* membrane fractions (mem+, mem–) showed no reaction; (D) dot blot analysis: intact induced and non-induced *E. coli_eno* transformants were diluted (log 2) and spotted onto a nitrocellulose membrane. The induced *E. coli_eno* transformants reacted with RαMsEno until a dilution of 1:8. The non-induced *E. coli_eno* transformants showed no reaction. M, molecular weight marker (kDa).

the protein with RαMsEno. A clear reduction of binding (43.13% ± 6.5% standard deviation) was measured compared to rMsEno without RαMsEno serum incubation.

3.6. *E. coli* transformants adhere to porcine RBCs

Adhesion of *E. coli_eno_gfp* was visualised by fluorescence microscopy. Alpha-enolase expressing *E. coli* transformants specifically adhered to porcine RBCs. The control (*E. coli_gfp*) showed no adhesion.

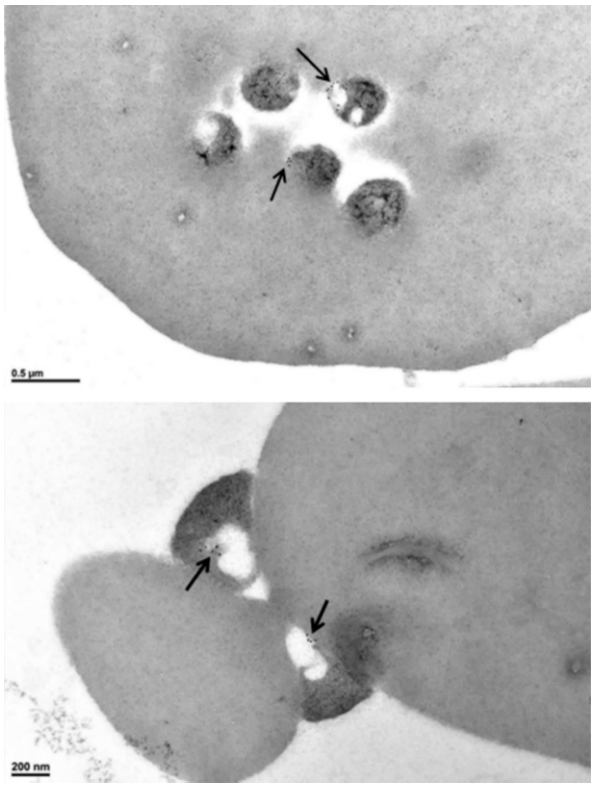

Fig. 3. Immunogold transmission electron microscopy demonstrated the surface-accessibility of *M. suis* α-enolase. Gold particle bound to *M. suis* α-enolase were accumulated on the attachment site between *M. suis* and porcine RBCs (indicated by arrows).

4. Discussion

Expression of virulence factors and attachment of bacteria to host cells, in the case of *M. suis* to porcine RBCs, are initial and crucial steps for the onset of disease. Until now, the identification of proteins which are involved in the adhesion of haemotrophic mycoplasmas and *M. suis* as well has been hampered mainly by the lack of *in vitro* cultivation systems and the related drawbacks.

Previously, a serological proteome analysis of *M. suis* purified from an acutely diseased pig and reconvalescent pig sera clearly indicated that *M. suis* α-enolase is an immunoreactive protein (Hoelzle et al., 2007c). For many infectious agents surface-displayed α-enolase has been described to function as adhesin (Giron et al., 1996; Pancholi, 2001; Pancholi and Chhatwal, 2003). Housekeeping enzymes such as α-enolase, glyceraldehyde-3-phosphate dehydrogenase or phospho-glycerokinase are supposed to carry out various additional and virulence-associated functions beside their common activities, i.e. in glycolysis (Pancholi and Chhatwal, 2003). Therefore, the present study aimed at the identification and characterisation of the *M. suis* α-enolase. Until now, one useful strategy for the investigation of putative *M. suis* adhesins and virulence factors was the screening of genomic *M. suis*

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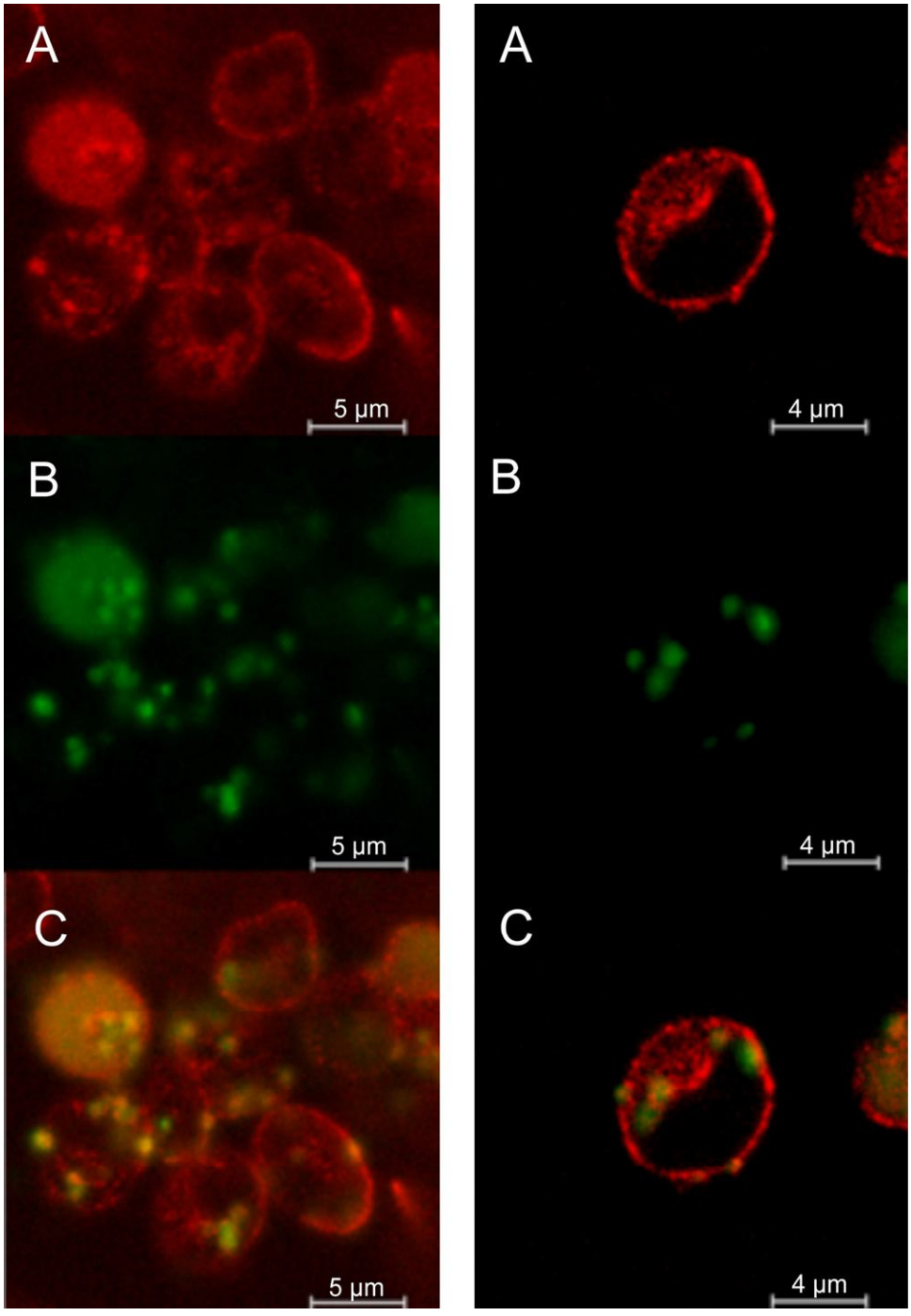

Fig. 4. Confocal laser scanning microscopy demonstrated the surface-accessibility of *M. suis* α-enolase. (A) TRITC-labelled porcine RBCs stained with glycophorin A (red fluorescence); (B) FITC-labelled *M. suis* cells stained with anti-enolase antibodies (RαMsEno; green fluorescence); (C) overlay of red and green fluorescence images clearly resulted in distinguishable green fluorescent dots demonstrating surface exposure of *M. suis* α-enolase.

DNA shotgun libraries (Hoelzle et al., 2007c,d). In doing so, we found a library clone (SG1-66) containing an incomplete ORF coded for the α-enolase protein of *M. suis*. By means of Southern blot hybridisation of genomic *M. suis* DNA the whole *α-enolase* nucleotide sequence of 1623 bp could be determined for *M. suis*. The characteristic enolase signature motif and the three functional sites for cofactor-binding (Mg^{2+}), substrate binding (PEP) and homodimerisation are present in the deduced aa sequence of *M. suis* α-enolase. Like that of *M. fermetans* or *Streptococcus pneumoniae* the *M. suis* α-enolase lacks a signal sequence or the typical motifs required for membrane anchoring

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(Bergmann et al., 2001; Yavlovich et al., 2007). The same phenomenon of surface localisation is described for MSG1, the surface-localised GAPDH-analogous adhesion protein of *M. suis* and for the GAPDH of *M. genitalium* (Alvarez et al., 2003; Hoelzle et al., 2007d). At this time it is supposed that these proteins form a new group of surface proteins which uses an as-yet-unknown mechanism for secretion and reassociation with the bacterial cell membrane/surface; they are often displayed on the cell surface in order to execute an infection-related function (Chhatwal, 2002; Pancholi, 2001; Pancholi and Fischetti, 1998). The clarification of the exact mechanism of translocation of intracellular located enzymes to the cell membrane should be the goal of further studies.

Interestingly, when compared to α-enolases of other related mycoplasmas, *M. suis* α-enolase possesses an extended C-terminus of about 90 aa. The protein in *M. suis* reacting with the RαMsEno showed a molecular mass of 59 kDa which corresponds to the predicted size based on the deduced aa sequence. Therefore, no obvious post-translational modification occurs. At the moment nothing is known about putative functions of this extension and, therefore, further investigations are necessary. We could suggest that the C-terminal tail might potentially allow *M. suis* α-enolase to interact with other proteins in the cytoplasm and overall *M. suis* to interfere with proteins of the porcine RBC membrane (due to its surface accessibility on the *M. suis* cell surface).

Our immunological data demonstrated that *M. suis* α-enolase exhibits strong immunogenicity. The recombinant protein in *E. coli* specifically reacted with sera of an *M. suis*-infected piglet, taken during the time course of the disease. Accordingly, in *Mycoplasma synoviae* for example, α-enolase has also been identified as one of its major immunogenic proteins (Bercic et al., 2008). To date, two recombinant proteins of *M. suis*, i.e. MSG1 and HspA1 are evaluated as diagnostic antigen (Hoelzle et al., 2007a,c,d). A third recombinant antigen in terms of the *M. suis* α-enolase could be an improving complement for the secure specific and sensitive serological diagnosis of *M. suis* infections in future.

Basically, proteins which are acting as potential adhesins have to be localised on the cell surface of pathogenic bacteria. By using immunoblot and immune electron as well as confocal laser scanning microscopic analyses, we determined the α-enolase in the cytoplasm, in the membrane, and on the surface of *M. suis*. Due to the surface expression of *M. suis* α-enolase, we supposed a multifunctional potential in addition to its common glycolytic activity in the cytoplasm. Since verification on the actual role of α-enolase in *M. suis* is still hampered by the lack of an *in vitro* culture system we used *E. coli* transformants as model system. In *E. coli* rMsEno was expressed in the cytoplasm as well as in the membrane. We further verified the surface accessibility of the rMsEno in the *E. coli* transformants by dot blot analysis. The fact that a cytoplasmic glycolytic protein is expressed on the surface of Gram-negative bacteria is out of the ordinary, especially due to the lack of classical signal sequences. However, the same phenomenon was found for the GAPDH-like *M. suis* protein MSG1 indicating a unique expression profile for *M. suis* (Hoelzle et al., 2007d). The found membrane localisation and the surface accessibility of α-enolase make evident that this protein has additional non-enzymatic functions in *M. suis*. The participation of α-enolase in the *M. suis* adhesion process to porcine RBCs was indicated by two factors: (i) non-adherent *E. coli* cells acquire the ability to bind to RBCs due to the expression of α-enolase on their surface and (ii) rMsEno binds to immobilised RBC lysate. This binding was significantly and specifically reduced by anti-α-enolase antibodies as well as by rMsEno. Further studies are needed to evaluate whether α-enolase could be part of an adhesion complex which is described for other mycoplasmas (Burgos et al., 2006; Chaudhry et al., 2007). Various proteomic studies with regard to the glycolytic pathway revealed that glycolytic enzymes often assembled in complexes in the cytoplasm to ensure higher glycolytic fluxes than non-interacting enzymes (Campanella et al., 2005; Dutow et al., 2010; Mowbray and Moses, 1976). Kuhner and co-workers proposed two glycolytic enzyme complexes in *M. pneumoniae* (Kuhner et al., 2009). One of these complexes is composed of GAPDH, Pgk, α-enolase, and chaperone protein DnaK. The presence of such complexation of *M. pneumoniae* glycolytic proteins was further supported by the findings of Dutow et al. (2010). They depicted α-enolase as the core protein that is capable of strongly interacting with all other glycolytic enzymes. It seems likely that similar glycolytic enzyme complexes around α-enolase might be formed in *M. suis* in order to act as complex adhesion apparatus. Since the *M. suis* GAPDH protein MSG1 was also described as a surface-localised adhesion protein, it might be suspected that both MSG1 and α-enolase participate in complexation-mediated adhesion (Hoelzle et al., 2007d).

5. Conclusion

In summary, we identified the 59 kDa *M. suis* α-enolase as a putative multifunctional glycolytic protein showing characteristics of adhesion to porcine RBCs. It is indispensable to identify the actual α-enolase binding partner on the porcine RBC, as well as other parts of a putative adhesion complex. The knowledge of this complex interplay between pathogen and host will allow a better understanding of the pathogenesis of *M. suis*-induced diseases and will help to develop prophylactic reagents, i.e. vaccines.

Conflict of interest statement

None of the authors of this paper has a financial personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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