Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in *Mycoplasma pneumoniae*

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The obligate pathogenic mycoplasma species *Mycoplasma pneumoniae* uses a limited but effective repertoire of virulence factors to infect and colonize the human respiratory tract. Besides the development of a unique adhesion complex and the expression of tissue-damaging factors, surface-located glycolytic enzymes and their capacity to bind to components of the human extracellular matrix (ECM) support pathogen–host interactions. Here, we demonstrated that the glycolytic enzymes enolase (Mpn606) and pyruvate dehydrogenase subunit B (Mpn392; PDHB) of *M. pneumoniae* show concentration-dependent binding to human plasminogen. Monospecific polyclonal antisera against both recombinant proteins reduced the binding to plasminogen significantly. The surface location of PDHB but not of enolase was demonstrated using Triton X fractionation of *M. pneumoniae* total protein content, membrane fractionation, colony blotting, mild proteolysis of mycoplasma cells, and immunofluorescence tests. To characterize the binding site of plasminogen in surface-displaced PDHB, the mycoplasmal protein was separated into four recombinant proteins followed by investigation of the binding behaviour of peptides that overlap the protein part interacting with plasminogen. Spot analysis resulted in a novel region of 12 amino acids (FPAMFQIFTHAA, position 91 to 102 of PDHB), which is responsible exclusively for binding of human plasminogen and also interacts in a dose-dependent manner with this host protein. The data indicate that the plasminogen-binding enzymes enolase and especially the surface-associated PDHB may contribute to the pathogenesis of *M. pneumoniae* infections.

INTRODUCTION

Epidemiological studies have shown that *Mycoplasma pneumoniae* is a common cause of a wide range of infections of the human respiratory tract (Waites & Talkington, 2004). Occurring mainly in children and young adults, the pathogen is responsible for not only up to 30% of community-acquired pneumonia cases but also for a broad spectrum of extrapulmonary complications (Narita, 2010).

Despite the fact that the cell wall-less species *M. pneumoniae* belongs to the smallest self-replicating microorganisms, with a genome size of about 816 kbp and 688 ORFs (Dandekar et al., 2000), this bacterium is remarkably adapted to the interaction with the respiratory epithelium of humans, the only known host. Nevertheless, the reduced genome minimizes the metabolic pathways used by *M. pneumoniae* (Kühner et al., 2009) and the repertoire of virulence factors which are expressed to support the parasitic lifestyle of the mycoplasmas. The most frequently investigated aspect of the interaction of *M. pneumoniae* with its host is the process of adherence to the respiratory epithelium as the first step in colonization. The formation of a unique tip-like structure comprising a complex of adhesins and adhesion-related proteins was shown to be the basis for effective adherence of bacteria to the respiratory mucosa (Krause & Balish, 2004). The further colonization process is accompanied by the release of hydrogen peroxide (Hames et al., 2009; Schmidt et al., 2011) and of the pertussis toxin-like CARDS toxin (Kannan & Baseman, 2006), resulting in vacuolization and destruction of epithelial cells.

Glycolytic enzymes are a further class of proteins which can be considered as virulence factors in the broader sense. Besides their primary role in glycolysis taking place in the cytosol of cells, these enzymes are transported by an unknown mechanism to the surface of micro-organisms. Here, these proteins interact with components of the human extracellular matrix (ECM) and of the human fibrinolysis system (Henderson & Martin, 2011). The dual role of glycolytic enzymes was first described in streptococci (Pancholi &
Fischetti, 1992). Meanwhile, these interactions have been reported for phylogenetically different micro-organisms, including fungi, parasites, and Gram-positive and Gram-negative bacteria (Barbosa et al., 2006; Bergmann et al., 2003; Egea et al., 2007; Gozalbo et al. 1998; Lama et al., 2009). Furthermore, an increasing number of microbial glycolytic enzymes have been characterized as involved in the interaction with human ECM, such as glyceraldehyde-3-phosphate dehydrogenases (Pancholi & Fischetti, 1992) and enolases (Pancholi & Fischetti, 1998). On the other hand, a broad spectrum of proteins of the human fibrinolysis system and ECM act as binding partners to glycolytic enzymes of prokaryotes, including plasminogen, fibronectin, fibrinogen, laminin, heparin and collagen (Pancholi & Chhatwal, 2003). Nevertheless, since the binding of metabolic enzymes to proteins of the human ECM has been described in numerous microbial species which are pathogens or commensals in many hosts and prefer different tissue systems for colonization, the mechanism can be considered as a common aspect in the micro-organism–host interaction.

Surface-associated proteins showing binding to human ECM have also been demonstrated in different members of the genus Mycoplasma including M. pneumoniae. Elongation factor Tu and pyruvate dehydrogenase subunit B of M. pneumoniae were reported as surface-located and function as binding partners to human fibronectin (Dallo et al., 2002). In a recent study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was characterized as interacting with human fibrinogen (Dumke et al., 2011). In addition, enolase of M. pneumoniae has been theoretically predicted to be a binding partner for human plasminogen (Chumchua et al., 2008). Other glycolytic enzymes of M. pneumoniae have been detected in the Triton X (TX)-100-insoluble protein fraction (Regula et al., 2001), which comprises the cytoskeleton-associated protein fraction of the bacterium. For example, fructose-bisphosphate aldolase was found among TX-100-insoluble proteins of M. pneumoniae and has been recently described in Neisseria meningitidis as surface-associated and involved in cytadherence (Tunio et al., 2010). Considering these aspects together, the occurrence of further interactions of glycolytic enzymes of M. pneumoniae with host components can be expected.

Plasminogen is a key protein of the human fibrinolysis system, interacting after conversion to plasmin with components of the ECM (Bergmann & Hammerschmidt, 2007). The aim of the present study was to investigate plasminogen-binding proteins in M. pneumoniae to contribute to an understanding of the interactions between the glycolytic enzymes of this clinically important mycoplasma species and proteins of the human ECM and the fibrinolysis system.

**METHODS**

**Bacteria and growth conditions.** M. pneumoniae strain M129 (ATCC 29342) was grown in 75 cm² cell culture flasks with 50 ml PPL0 medium (Becton Dickinson) supplemented with 20% horse serum (Gibco), 10% yeast extract (Gibco) and 1.5% glucose (Merck) at 37 °C. After a change in the colour of the medium from red to orange (equivalent to approximately 10³ to 10⁴ c.f.u. ml⁻¹), attached cells were washed twice with PBS (8.8 g NaCl l⁻¹, 1.7 g Na₂HPO₄ l⁻¹, 0.2 g NaH₂PO₄ l⁻¹, pH 7.2) and resuspended with a cell scraper in 2 ml of the same buffer.

To produce recombinant proteins, the Escherichia coli strains NovaBlue and BL21 (DE3; both Novagen) were cultured in Luria–Bertani (LB) broth (Becton Dickinson) with kanamycin (25 μg ml⁻¹; Sigma) at 37 °C with agitation (200 r.p.m.).

**TX fractionation of M. pneumoniae total proteins and membrane purification of M. pneumoniae cells.** To localize proteins in the mycoplasma cell, bacteria were treated with the detergents TX-100 and TX-114. TX-100-insoluble proteins represent cytoskeleton-associated components (defined as Triton shell). Cytosplasmic components are detected mainly among the TX-100-soluble and the aqueous phase proteins after TX-114 treatment, whereas membrane proteins can be found in the TX-114 detergent phase and in the TX-114-insoluble fraction (Prof & Herrmann, 1994).

**M. pneumoniae** cells were grown as described previously (Dumke et al., 2011). Harvested bacterial suspension with a protein content of 1.5 mg was diluted in 800 μl PBS with protease inhibitor (Roche Diagnostics). Cell agglomerations were reduced by resuspending the bacteria with a 27G syringe, and 200 μl TX-114 (10%, Sigma) was added. Extraction of proteins was carried out as described elsewhere (Wise et al., 1995). Briefly, after incubation for 2 h at 4 °C, the suspensions were pelleted by centrifugation at 13,000 g (10 min, 4 °C). The supernatant was transferred into a fresh reaction tube. The pellet, which corresponds to the M. pneumoniae TX-insoluble protein fraction, was resuspended in 1 ml PBS with protease inhibitor. After an incubation period of 5 min at 37 °C, the supernatant was centrifuged at 13,000 g (3 min). The upper aqueous phase and the lower detergent phase were transferred to a fresh reaction tube. TX-114 (200 μl) was added to the aqueous phase and 800 μl PBS to the detergent phase. The suspensions were incubated for 5 min at 4 °C and for 5 min at 37 °C. A centrifugation step (13,000 g, 3 min) separated the two phases. This washing step was repeated three times. The protein fractions were adjusted to 1 ml with PBS, aliquoted and stored at −20 °C.

In addition, the same amount of M. pneumoniae cells was also treated with 2% (v/v) TX-100 (Sigma) as described by Stevens & Krause (1991) to separate the TX-100-insoluble and the TX-100-soluble protein fractions. Both fractions were adjusted to 1 ml with PBS, aliquoted and stored at −20 °C.

In addition, membranes of 2.5 mg freshly grown M. pneumoniae cells were isolated as described elsewhere (Prof & Herrmann, 1994). Briefly, bacterial cells were dissolved in 1 ml HPLC water and disrupted by sonication. Undisrupted cells were removed by low-speed centrifugation (6,000 g, 15 min, 4 °C). Membranes were separated from cytosolic proteins by ultracentrifugation (120,000 g, 1 h, 4 °C) and suspended in 300 μl PBS with protease inhibitor. Cytosolic fraction was concentrated by Vivaspin 6 columns [molecular weight cut-off (MWCO) 5000; Sartorius] to 300 μl. Protein concentrations of both fractions were determined with the BCA Protein Assay kit (Pierce).

**Binding of human plasminogen to M. pneumoniae whole cells.** The total protein content of freshly grown M. pneumoniae cells (150 μg) was separated by SDS-PAGE using 10% NuPage Bistris gels (Invitrogen) and blotted onto a nitrocellulose membrane. Human plasminogen (40 μg ml⁻¹, Sigma) and PBS (control) were added to the membranes and incubated for 2 h at room temperature with gentle agitation. After three washings with 10% PCS in PBS/Tween
for 10 min, rabbit antiserum against human plasminogen (1:500, Sigma) was added to both membranes and incubated for 1.5 h at room temperature. Bound plasminogen was detected with peroxidase-conjugated anti-rabbit IgG (1:1000, Sigma). Finally, the membranes were incubated with 4-chloro-1-naphthol (Serva) in the presence of hydrogen peroxide.

Binding of plasminogen to *M. pneumoniae* complete protein fractions, TX-114-insoluble and cytosolic proteins was quantified by ELISA as reported recently (Dumke *et al.*, 2011). Briefly, Microlon plates (Greiner) were coated with 0.25 μg (in 50 μl 0.05 M carbonate buffer, pH 9.6) of the three protein fractions per well. After first blocking (10% FCS in PBS), wells were incubated with different plasminogen concentrations (0.5, 5.0 and 50.0 μg ml⁻¹ in PBS) and PBS for 1.5 h (room temperature). The wells were washed three times. Bound plasminogen was detected with rabbit anti-plasminogen (1:250) and peroxidase-conjugated anti-rabbit IgG (1:500). Substrate (TMB Super Slow, Sigma) was added, the reaction was stopped with 1 M HCl and the OD₄₅₀ was measured with a reference wavelength of 620 nm. Carbonate buffer without antigen was used as negative control and the optical density values obtained were subtracted from the optical density of wells containing the antigens.

**Expression and purification of recombinant proteins.** Freshly grown *M. pneumoniae* cells were harvested as described above and the DNA was prepared with the QIAamp DNA Mini kit (Qiagen) as recommended by the manufacturer. In a first PCR, the genome regions surrounding the two genes of interest were amplified with the primers MpPdhBf1/r and MpEnof1/r (Table 1). These PCR products (1:10 diluted in HPLC water) were used as targets for the multiple mutation reaction (MMR; Hames *et al.*, 2005) to amplify the complete genes and to mutate simultaneously the single TGA codon (encoding tryptophan in mycoplasmas; Inamine *et al.*, 1990) occurring in both genes. Using *pfu* polymerase (Fermentas), the primer pairs MpPdhBVf/Vr and MpEnoVf/Vr create vector-specific overhangs, whereas the mutation primers MpPdhBM and MpEnoM change the TGA codons into TGG. The MMR products were used to produce a ligation-independent annealing product with the pET-30/LIC vector (Novagen) as described by the manufacturer. After transformation of *E. coli* NovaBlue and BL21(DE3), the vector in selected *E. coli* colonies was sequenced to check the correct insertion of the complete gene in the pET vector and the exchange of the TGA codons (for primers see Table 1, including vector-specific primer ExpNF).

Expression and purification of the N-terminal 6xHis-tagged recombinant proteins were performed as described (Dumke *et al.*, 2011). The recombinant proteins were concentrated by vivaspin 6 columns (MWCO 5,000) and the protein concentration was measured with the BCA protein assay kit.

### Table 1. Oligonucleotides used in the study

Underlined sequences, vector-specific sequence; italic sequences, mutation of TGA to TGG.

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<th>Target</th>
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<td>Amplification and sequencing</td>
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<tr>
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<td>MpPdhBVf</td>
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*Hames *et al.* (2005).
To confirm the quality of recombinant protein expression, the protein eluates were analysed by SDS-PAGE as described above, followed by Coomassie staining (Merk). For immunoblotting, the concentrated recombinant proteins were transferred to nitrocellulose membranes. Protein detection was carried out with anti-His-tag monoclonal mouse antibody (1:500, Sigma) and peroxidase-conjugated anti-mouse IgG (1:1000, Pierce). The occurrence of antibodies against pyruvate dehydrogenase subunit B (PDHB) and enolase of *M. pneumoniae* in an infected host was tested after incubation of blotted recombinant proteins with an antiseraum (1:250) obtained after threefold intranasal infection of a guinea pig with *M. pneumoniae*. Detection was done with anti-guinea pig immunoglobulin (1:1000, Sigma).

Production of guinea pig polyclonal antibodies. Anaesthetized guinea pigs were immunized subcutaneously with 100 μg recombinant PDHB or enolase from *M. pneumoniae* in 200 μl PBS mixed with 100 μl complete Freund’s adjuvant (Sigma). This was followed after 4 weeks by two booster immunizations at intervals of 1 week using incomplete Freund's adjuvant (Sigma). Ten days after the last immunization, the animals’ blood was collected by heart aspiration and serum fractions were prepared. To remove antibodies against the remaining proteins of *E. coli* BL21(DE3) in the antigen preparation, the sera obtained were incubated overnight at 4 °C with sonicated and immobilized *E. coli* BL21(DE3) whole-cell extract as described previously (Dumke et al., 2011), aliquoted, and stored at −20 °C. The reactivity of the sera (1:500) was tested with the total protein content of *M. pneumoniae* by immunoblotting and ELISA, and compared with the antisera obtained after threefold intranasal infection of guinea pigs with *M. pneumoniae*.

Surface location of *M. pneumoniae* PDHB and enolase. Since the localization of proteins is a crucial aspect of the study, we used different methods to investigate their possible exposure on the surface of mycoplasma cells. For ELISA experiments, wells were coated with the TX-100-insoluble and TX-100-soluble as well as with the TX-114-insoluble, TX-114-soluble (detergent) and aqueous fractions of *M. pneumoniae* total cell proteins, as described above. Dilution of antigens obtained after TX extractions was equivalent to compare the quantitative occurrence of the two glycolytic enzymes in the different protein fractions. Detection was performed with sera against PDHB and enolase diluted 1:250 followed by anti-guinea pig IgG (1:500). To control possible cross-contamination of protein fractions, rabbit anti-HMW1 (adherence-related protein occurring in the TX-100- and TX-114-insoluble fraction) and anti-elongation factor G (EF-G, cytosolic protein) sera were used (Proft & Herrmann, 1994; Regula et al., 2001). For confirmation of the result, the recombinant proteins PDHB and enolase (10 μg ml⁻¹ each) were used as antigens for coating, and were incubated with rabbit antibodies against the TX-100-insoluble or against the cytosolic fractin of the *M. pneumoniae* whole-cell extract (1:250 each) and anti-rabbit IgG (1:500). In addition, the separated membrane and cytosolic fractions were diluted in a similar way and used as antigens in ELISA experiments. Detection of PDHB and enolase was carried out with anti-PDHB or anti-enolase (1:500) and anti-guinea pig immunoglobulin (1:1000).

To further assess surface-localized regions of the two proteins, PDHB and enolase, mild surface proteolysis of *M. pneumoniae* cells was carried out. Mycoplasmas were grown as described above and harvested, and the protein concentration was adjusted to 200 μg ml⁻¹. The cells were centrifuged (13 000 g, 5 min) and treated with 5 and 20 μg trypsin (Sigma) in PBS for 30 min at 37 °C. After centrifugation for 10 min (13 000 g), the pellet was resuspended in 100 μl protein sample buffer and boiled for 10 min at 100 °C. The total proteins were separated by SDS-PAGE and blotted to a nitrocellulose membrane as described above. The membranes were incubated with antisera (1:500) against PDHB and enolase, and as controls against EF-G and the surface-located, C-terminal part of the main adhesin P1 of *M. pneumoniae* (Schurwanz et al., 2009). Anti-rabbit IgG (EF-G) and anti-guinea pig IgG (both 1:500) were used to detect the proteins.

As a further method, the colony blot technique was used to demonstrate the presence of PDHB and enolase on the surface of *M. pneumoniae* cells. Freshly grown mycoplasmas were harvested as described above, serially diluted 10 times with PPLO broth and spread out (100 μl each) in a Petri dish containing PPLO agar. The plates were incubated for 10 days at 37 °C. After choosing the colonies with an appropriate number of colonies (200 to 1000), the mycoplasmas were covered with pre-cut pieces of nitrocellulose membrane for 5 min. The membranes were dried for 10 min at room temperature, washed, and blocked as described above. The reactivity of the colonies was tested with anti-PDHB, anti-enolase or anti-EF-G (1:250), followed by incubation with peroxidase-conjugated anti-guinea pig or anti-rabbit IgG (1:500).

Alternatively, the cell surface localization of both of the glycolytic proteins was investigated by fluorescence microscopy. Freshly grown *M. pneumoniae* cells were harvested, and 1 ml of the bacterial suspension (diluted 1:10 with PPLO broth) was inoculated in each of the four cavities of a cover slide (Nunc) to grow for 2 days at 37 °C. After removing the supernatant, the cells were fixed with formaldehyde (10% in PBS, Merck) for 20 min and blocked for 30 min at 37 °C. The following mixtures of antisera (1:250 each) were added to the cavities: guinea pig and rabbit pre-immune sera (negative control), guinea pig anti-total protein and rabbit anti-TX-100-insoluble fraction of total proteins (positive control), guinea pig anti-PDHB and rabbit anti-TX-100-insoluble fraction of total proteins (PDHB), and guinea pig anti-enolase and rabbit anti-TX-100-insoluble fraction of total proteins (enolase). After an incubation period of 1 h at 37 °C, the cells were washed three times, a mixture of FITC-labelled anti-guinea pig IgG (Sigma) and tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC)-labelled anti-rabbit IgG (Sigma) in a dilution of 1:500 was added, and the cover slides were incubated for 30 min at 37 °C in the dark. The cells were washed and observed under a fluorescence microscope (Zeiss) at ×400 magnification.

Influence of anti-PDHB and anti-enolase sera on binding of human plasminogen to *M. pneumoniae* whole cells, recombinant PDHB and recombinant enolase. Total proteins of *M. pneumoniae*, and the recombinant proteins PDHB and enolase, were used to coat wells of ELISA plates as described above. To characterize the ability of the specific antisera against PDHB and enolase to reduce binding of plasminogen to these antigens, different plasminogen concentrations (1–100 μg ml⁻¹) were incubated with anti-PDHB and anti-enolase sera (both 1:100) and with PBS for 1.5 h at room temperature in an overhead shaker. The plasminogen-serum/PBS solutions were added to the immobilized total *M. pneumoniae* proteins as well as to the recombinant proteins and incubated for 1.5 h at room temperature. After washing three times, bound plasminogen was detected with anti-plasminogen (1:250) and peroxidase-conjugated anti-rabbit IgG (1:500) as described above.

Characterization of the plasminogen-binding region in *M. pneumoniae* PDHB. The complete PDHB protein was divided into four parts (amino acids 1–82, 83–164, 165–246 and 247–327; Table 1) and recombinant proteins were produced as described above. Purified and concentrated recombinant proteins (100 μg each) were analysed by SDS-PAGE and blotted. Membranes were incubated with human plasminogen (40 μg ml⁻¹) and with PBS as control. Bound plasminogen was detected with anti-human plasminogen (1:500) and anti-rabbit IgG (1:500). The plasminogen-binding recombinant protein PDHB-2 was divided into 19 peptides, each consisting of 12 amino acids with an overlap of three amino acids, and synthesized (Centic Biotec). The peptides (1 mg ml⁻¹) were spotted onto two
Expression and purification of *M. pneumoniae* enolase and PDHB, and generation of polyclonal antibodies

Comparison of the molecular mass of the plasminogen-binding proteins with those of mycoplasma glycolytic enzymes provided an indication that enolase (49.2 kDa, as theoretically predicted; Chumchua *et al.*, 2008) and PDHB (35.9 kDa) are possible binding partners. To test this hypothesis, single TGA codons in both encoding genes were exchanged to circumvent the stop of translation, and the complete genes were cloned into the pET-30 vector. The proteins were overexpressed in *E. coli*, purified by Ni-agarose affinity chromatography and analysed by SDS-PAGE. Coomassie staining of the gels resulted in two prominent protein bands with the expected molecular mass (Fig. 2a). The blotted recombinant proteins enolase and PDHB were further characterized by detection with monoclonal anti-His antibodies (Fig. 2b). Interestingly, the antiserum of a guinea pig immunized three times with *M. pneumoniae* cells subcutaneously gave a positive signal with PDHB, indicating the production of specific antibodies (Fig. 2b). In contrast, the reaction of the same serum with recombinant enolase remained negative.

The purified recombinant proteins were used to generate guinea pig polyclonal antisera. The specificity of the
antibodies obtained was tested with blotted total proteins of *M. pneumoniae*. After the immune reaction, nearly exclusive signals were found at molecular masses matching the predicted molecular masses of mycoplasma PDHB and enolase (Fig. 2c). Using total *M. pneumoniae* proteins as antigen, ELISA reactivities of anti-PDHB and the antiserum against total proteins of *M. pneumoniae* resulted in comparable optical density values, whereas the anti-enolase serum reacted significantly more weakly (Fig. 2d). Nevertheless, the occurrence of detectable concentrations of both proteins among the total protein content of *M. pneumoniae* was confirmed.

**Localization of enolase and PDHB**

To investigate the cellular distribution of the two proteins, we tested different approaches. The ELISA reaction of *M. pneumoniae* PDHB with antibodies to the TX-insoluble and to the cytosolic fractions confirmed the presence of this glycolytic enzyme in both compartments of the mycoplasma cell (Fig. 3a). Using equivalent amounts of different protein fractions as antigens, anti-PDHB reacted strongly with the TX-100- and TX-114-insoluble proteins as well as with the TX-100-soluble and the aqueous phase proteins after TX-114 fractionation (Fig. 3b). Therefore, besides the occurrence in the cytosol, PDHB could be found among the components of the cytoskeleton. In contrast, enolase was detected in the cytosolic protein fraction, suggesting the absence of the enzyme among the cytoskeleton- and membrane-associated proteins of the mycoplasmas. The control sera reacted exclusively with the TX-100- and TX-114-insoluble proteins (anti-HMW1) or with the TX-100-soluble/aqueous phase proteins (anti-EF-G), confirming a minor cross-contamination between the fractions. The results were confirmed by ELISA experiments using the membrane and cytosolic fractions of *M. pneumoniae* proteins as antigens (Fig. 3c). No significant differences of optical density values were found after incubation with anti-PDHB, suggesting comparable PDHB concentrations in both fractions. In contrast, the reactivity of the membrane

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**Fig. 2.** Characterization of recombinant PDHB and enolase of *M. pneumoniae* and the derived polyclonal guinea pig antisera. (a) SDS-PAGE of purified and concentrated recombinant proteins followed by Coomassie staining. (b) Blotted purified and concentrated recombinant proteins (100 μg each) incubated with monoclonal anti-His antibody (lane 1) and guinea pig serum obtained after three intranasal infections of the animals with freshly grown *M. pneumoniae* M129 cells (lane 2). (c) Reaction of antisera obtained after subcutaneous immunization of guinea pigs with recombinant PDHB and enolase of *M. pneumoniae*. Incubation of blotted whole-cell extract of *M. pneumoniae* M129 with both antisera followed by a peroxidase-labelled anti-guinea pig IgG secondary antibody. (d) Results of ELISA experiments to compare the reactivity of the guinea pig antisera. Plates were coated with the total protein content of *M. pneumoniae* M129 and probed with the antiserum obtained after three intranasal infections of an animal (anti-Mp), and with antisera to recombinant PDHB and to recombinant enolase. Data represent mean and SD of eight parallel experiments.
Fig. 3. Surface localization of PDHB and enolase on *M. pneumoniae* cells. (a) Reactivity of the rabbit anti-TX-100-insoluble protein fraction and anti-cytosolic protein fraction of *M. pneumoniae* with immobilized recombinant proteins PDHB and enolase, as measured by ELISA. (b) ELISA reaction of guinea pig anti-PDHB and anti-enolase with the immobilized fractions after TX-100 and TX-114 extraction of total proteins of *M. pneumoniae* M129 (mean and SD of eight parallel experiments). Anti-HMW1 (TX-insoluble protein) and anti-EF-G (cytosolic protein) sera were used as controls. (c) Membrane association of PDHB and enolase analysed by ELISA (mean and SD of eight parallel experiments). (d) Results of trypsin digestion of freshly grown *M. pneumoniae* cells. Trypsin-treated and untreated total proteins were blotted and incubated with anti-PDHB, anti-enolase, anti-P1 (C terminus, positive control) and anti-EF-G serum (negative control). (e) Reactivity of 10 day-old colonies of *M. pneumoniae* with anti-PDHB serum. Bar, 200 μm.
fraction of total proteins with anti-enolase remained below 0.1.

After treatment of intact bacteria with trypsin, weak digestion products of PDHB were observed without complete disappearance of full-length protein, demonstrating partial accessibility of PDHB to trypsin (Fig. 3d). Enolase was found to be stable under these experimental conditions. Proteolysis of the cell surface led to complete digestion of the C-terminal part of the membrane-spanning P1 adhesin (positive control), whereas the cytosolic EF-G was not influenced, indicating that bacterial membrane integrity was maintained during the treatment.

In colony blot experiments, anti-PDHB reacted with immobilized proteins from the surface of mycoplasma colonies (Fig. 3e), whereas no reactivity with anti-enolase could be demonstrated (data not shown). The incubation of blots with anti-EF-G was also tested and remained negative, which suggests that cytosolic proteins are not detected under the experimental conditions (data not shown).

Freshly grown mycoplasma cells were fixed and treated with guinea pig anti-PDHB and anti-enolase sera. The sera were mixed with rabbit antiserum against TX-100-insoluble proteins as a positive control serum that reacts with many surface-exposed, membrane-associated proteins of M. pneumoniae, e.g. the adhesins (Regula et al., 2001). The detected proteins were visualized by fluorescence microscopy using FITC- or TRITC-labelled secondary antisera against guinea pig and rabbit immunoglobulins, which allow evaluation of the test procedure (Fig. 4). Incubation of the cells with guinea pig anti-PDHB serum resulted in clear signals, indicating a surface localization of the protein. No signals were obtained after treatment of the slide with anti-enolase and the FITC-labelled anti-guinea pig secondary antibody. Reactivity of TX-100-insoluble proteins with the bacteria in this slide was demonstrated by TRITC-labelled anti-rabbit IgG. The negative control (mixture of guinea pig and rabbit pre-immune sera) confirmed the limited occurrence of cross-reactions of both secondary antibodies used. In addition, the reaction of fixed M. pneumoniae cells with anti-EF-G was pre-tested and found to be negative (data not shown).

Taking the results together, a cytosolic and a surface localization of M. pneumoniae PDHB can be assumed, whereas the enolase occurred mainly amongst the cytosolic proteins of M. pneumoniae cells.

**BINDING OF HUMAN PLASMINOGEN TO M. PNEUMONIAE WHOLE CELLS, RECOMBINANT ENOLASE AND RECOMBINANT PDHB**

ELISA experiments confirmed the concentration-dependent binding of plasminogen to all of the three antigen preparations (Fig. 5a–c). In contrast to treatment of plasminogen with PBS, the pre-incubation of plasminogen with anti-PDHB as well as with anti-enolase reduced the binding significantly. The weakest reduction was observed in the interaction of plasminogen with total M. pneumoniae proteins, whereas the binding to the recombinant proteins was blocked to a greater extent by both of the specific antibodies. Furthermore, pre-incubation of plasminogen

![Fig. 4. Reactivity of anti-PDHB and anti-enolase of M. pneumoniae as determined by immunofluorescence. Fixed mycoplasma cells were probed with a mixture of guinea pig and rabbit pre-immune sera (negative control), a mixture of guinea pig anti-total protein content and rabbit anti-TX-100-insoluble proteins of M. pneumoniae (positive control), a mixture of guinea pig anti-PDHB and rabbit anti-TX-100-insoluble proteins (PDHB), and a mixture of guinea pig anti-enolase and rabbit anti-TX-100-insoluble proteins (enolase). After washing, cells were incubated with a mixture of FITC-conjugated anti-guinea pig IgG and TRITC-conjugated anti-rabbit IgG. Bar, 10 μm.](http://mic.sgmjournals.org)
Characterization of the plasminogen-binding region in *M. pneumoniae* PDHB

To define the plasminogen-binding site of the 36 kDa protein (328 amino acids), PDHB was divided into four parts, which were overexpressed in *E. coli* and purified as described above (Fig. 6a). After immunoblotting with anti-PDHB polyclonal serum, strong signals were found, confirming the specificity of the four recombinant proteins tested (data not shown). Only the blotted recombinant protein PDHB-2 reacted with human plasminogen (Fig. 6b). Negative control incubation of the blot with PBS followed by the same immune reaction confirmed that the reaction of the recombinant protein is caused by the interaction with plasminogen. Dot blot testing of 19 synthetic peptides overlapping the PDHB protein region from amino acids 83 to 164 (corresponding to recombinant protein PDHB-2) resulted in the exclusive reactivity of peptide P3 with human plasminogen (Fig. 6c). Strong signals from the positive controls, complete recombinant protein PDHB and *M. pneumoniae* total protein, confirmed the interaction with plasminogen. Analysing the negative control membrane (same antigens and immune reaction but incubation with PBS instead of plasminogen), no signals were obtained for any peptide or either control (data not shown). In a range from 1 to 200 µg, human plasminogen bound in a concentration-dependent manner to peptide P3 immobilized in the wells of an ELISA plate (Fig. 6d). Pre-incubation of plasminogen with PDHB diluted 1 : 500 and pre-immune guinea pig serum resulted in a weak but statistically significant reduction of PDHB binding (Fig. 6e). It should be noted that in ELISA experiments testing the reactivity of the panel of PDHB peptides with polyclonal anti-PDHB, high optical density values were demonstrated for peptide P17 only (data not shown).

**DISCUSSION**

As bacteria with a parasitic lifestyle in humans and animals, members of the genus *Mycoplasma* such as *M. pneumoniae* use different mechanisms to interact with host tissues, mainly via mucosal surfaces. Effective adherence to target cells is the first step in reaching preferred niches, and this is followed by access to nutrient conditions that allow growth and multiplication. Within these multi-factorial processes, proteins on the mycoplasma surface play a central role in the host–pathogen interaction. In this context, binding to host ECM components is mediated by different mycoplasma molecules, such as adhesins and proteins of unknown function in *Mycoplasma gallisepticum* (Jenkins et al., 2007; May et al., 2006) and *Mycoplasma hyopneumoniae* (Burnett et al., 2006; Seymour et al., 2010), as well as proteins involved in protein synthesis and housekeeping enzymes in other species (Chen et al., 2011; Dallo et al., 2002; Dumke et al., 2011; Hoelzle et al., 2007). In addition to the described association of PDHB and elongation factor Tu of *M. pneumoniae* with human fibronectin (Dallo et al., 2002) and of GAPDH with human fibrinogen (Dumke
et al., 2011), here we show that PDHB and enolase are binding partners of human plasminogen. Furthermore, the present study confirmed the results of previous studies indicating the occurrence of multi-functional binding molecules as shown for PDHB, which interacts with fibronectin as well as with plasminogen. It can be assumed that this aspect is of special importance in a microorganism with a greatly reduced genome, where the ability of a protein to play a dual role in metabolism and in the interaction with the host might help to compensate for the limitations of reduced genetic resources (Dallo et al., 2002).

Glycolysis is the major pathway in *M. pneumoniae* and other mollicutes for energy production by substrate phosphorylation. Interactions between glycolytic enzymes optimize the process, in which enolase has been characterized as a core protein interacting with other enzymes of glycolysis (Dutow et al., 2010). Most of the proteins involved in the pathway are phosphorylated (Schmidl et al., 2010), suggesting that their activity and/or localization may be regulated. The data of the present study demonstrate that two glycolytic enzymes of *M. pneumoniae* bind to human plasminogen. The occurrence of more than a single microbial molecule interacting with a given ECM component has been reported for different species such as *Streptococcus pneumoniae* (Bergmann et al., 2001, 2004) and *Candida albicans* (Crowe et al., 2003). The identification of *M. pneumoniae* enolase as a binding partner of plasminogen confirmed the theoretical prediction based on homology modelling (Chumchua et al., 2008) and was in accordance with experimental results from the mycoplasma species *Mycoplasma fermentans* (Yavlovich et al., 2007), *M. gallisepticum* (Chen et al., 2011) and *Mycoplasma suis* (Schreiner et al., 2012). Besides GAPDH, enolase is one of most frequent members of the class of surface-localized glycolytic enzymes, as described in phylogenetically different species such as *Aeromonas hydrophila* (Sha et al., 2009), *Bacillus anthracis* (Agarwal et al., 2008), *Borrelia burgdorferi* (Nogueira et al., 2012), *Bifidobacterium* sp. (Candela et al., 2009), *C. albicans* (Jong et al., 2003), *Lactobacillus plantarum* (Castaldo et al., 2009), *Paracoccidioides brasiliensis* (Donofrio et al., 2009), *Staphylococcus aureus* (Carneiro et al., 2004), *S. pneumoniae* (Bergmann et al., 2001) and *Trichomonas vaginalis* (Mundodi et al., 2008). In most cases, enolases interact with plasminogen but binding to fibronectin and laminin has also been reported (Carneiro et al., 2004; Castaldo et al., 2009; Donofrio et al., 2009). The *M. pneumoniae* enolase shows typical putative plasminogen-binding sites, such as lysine in the C terminus, 448^K^FKNIK^452^ and a lysine-rich internal motif, 268^KRYVFKKGIKI^284 (Bergmann et al., 2003; Derbise et al., 2004; Yavlovich et al., 2007). A surprising fact was the absence of this enzyme on the surface of mycoplasma cells. *M. pneumoniae* enolase is a protein of 456 aa, having identities of 54.5, 56.2 and 64.4 % to the surface-associated homologues in the Mycoplasma species *M. suis* (540 aa), *M. fermentans* (455 aa) and *M. gallisepticum* (476 aa) that have so far been characterized. Since the glycolytic enzymes investigated lack the cleavable N-terminal signal sequence and were secreted in a non-classical way, the molecular basis of differences in the localization pattern of proteins with identical function is not understood. As described for elongation factor Tu of *Mycoplasma genitalium* and *M. pneumoniae* (the amino acid sequence is 96 % identical), small sequence differences will influence the binding pattern to ECM components (Balasubramanian et al., 2009). It is possible that this influences the mechanism(s) involved in the transport of the protein to the surface of bacteria.

The occurrence of *M. pneumoniae* PDHB in high concentrations in the TX-100-insoluble fraction of total proteins (Regula et al., 2001), and its localization on the surface of mycoplasma cells as demonstrated by immunogold electron microscopy and whole cell radio-immunoprecipitation (Dallo et al., 2002), have been reported. Using proteome analysis, PDHB was identified as an antigen that reacts with *M. pneumoniae*-positive patient sera (Nuyttens et al., 2010). The interaction of PDHB with plasminogen and the previously described binding to human fibronectin (Dallo et al., 2002) show that this molecule belongs to the group of proteins mediating contact to more than one ECM component, such as enolase of *P. brasiliensis* (Donofrio et al., 2009; Nogueira et al., 2010). To our knowledge, this is the first proof of an interaction of PDHB with human plasminogen. The identification of the plasminogen-binding region of 12 aa in PDHB offers new insights into the mechanism of interaction between the two partners. The binding region (amino acids 91–102) is located directly after the predicted transmembrane domain of the protein (www.cbs.dtu.dk/services/TMHMM/). *M. pneumoniae* PDHB shows a C-terminal lysine which was characterized as a potential binding region in enolase (Derbise et al., 2004), but no interaction of the corresponding recombinant protein derived from this region with human plasminogen was found. In contrast, the plasminogen-binding peptide identified has no lysine, indicating lysine-independent interactions between the two proteins. This is in accordance with findings from *C. albicans* that confirm the existence of several glycolytic enzymes with plasminogen-binding properties without C-terminal lysine residues (Crowe et al., 2003).

This raises the question of whether sera against PDHB and enolase of *M. pneumoniae* will decrease the adherence of mycoplasmas to cells, as reported for enolase of *M. gallisepticum* (Chen et al., 2011). Pre-incubation of freshly grown and FITC-labelled *M. pneumoniae* cells with anti-PDHB and anti-enolase did not reduce their adhesion to HeLa cells (data not shown), as measured by a FACS-based quantitative adherence inhibition assay (Schurwanz et al., 2009). Similar results were obtained with the antiserum to GAPDH (Dumke et al., 2011), and indicate that surface-located glycolytic enzymes of *M. pneumoniae* are not involved in the primary adhesion process and/or that small, non-antigenic protein regions are responsible for interaction with components of the human ECM. This is supported by the finding of the limited (12 aa) PDHB region that binds...
plasminogen and shows no detectable immune reaction with polyclonal anti-PDHB. In line with the absence of a surface association of enolase, anti-enolase antibodies were not detected in the serum of an animal infected three times with *M. pneumoniae*. In contrast, recombinant PDHB reacts with this serum, further supporting the exposure of this protein on the surface of mycoplasma cells. Taken together, the well-defined complex of adhesins and adhesion-related proteins which is described in *M. pneumoniae* (Krause & Balish, 2004) accomplishes the adherence of mycoplasmas to
human epithelial cells as the first step of the infection process. Since antibodies against surface-localized glycolytic enzymes have no influence on adherence, it can be speculated that the interaction of these proteins with host components is important in further stages of colonization. This includes protection against the host’s immune response, as described for Streptococcus spp. (Feng et al., 2009; Madureira et al., 2007), adherence to deeper layers of epithelium showing higher concentrations of ECM molecules (Yavlovich et al., 2004), and access of the bacteria to more favourable nutrient conditions.

In conclusion, with enolase and PDHB we identified two M. pneumoniae proteins that bind to human plasminogen. The results of this study provided a further piece in the puzzle of the interactions of glycolytic enzymes of the obligate pathogenic species M. pneumoniae with human ECM and fibrinolysis components. In recent years, it has become obvious that the dual role of these enzymes is a key aspect in understanding the interaction of many pathogens with their hosts. Especially with M. pneumoniae, one of the smallest known self-replicating human pathogens, further investigations can help to provide a comprehensive picture of the mechanisms involved in the overall success of this species in colonizing the host respiratory tract.

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