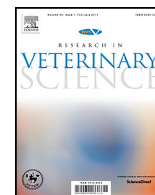
Research in Veterinary Science 97 (2014) 282–287

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc



Interactive host cells related to *Mycoplasma suis* α-enolase by yeast two-hybrid analysis

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ARTICLE INFO

Article history:
Received 18 February 2014
Accepted 3 July 2014

Keywords:
Mycoplasma suis
α-Enolase gene
Yeast two-hybrid
Interactive cellular protein
Porcine infections

ABSTRACT

Mycoplasma suis belongs to the haemotrophic mycoplasmas, which colonise the 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*. In addition to MSG1 protein, α-enolase is the second adhesion protein of *M. suis*, and may be involved in the adhesion of *M. suis* to porcine red blood cells (RBC). To simulate the environment of the RBC, we established the cDNA library of swine peripheral blood mononuclear cells (PBMC). The yeast two-hybrid (Y2H) system was adopted to screen α-enolase interactive proteins in the PBMC line. Alignment with the NCBI database revealed four interactive proteins: beta-actin, 60S ribosomal protein L11, clusterin precursor and endonuclease/reverse transcriptase. However, the *M. suis* α-enolase interactive proteins in the PBMC cDNA library obtained in the current study provide valuable information about the host cell interactions of the *M. suis* α-enolase protein.

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1. Introduction

Mycoplasma suis (*M. suis*), a member of the uncultivable haemotrophic mycoplasmas, may be found as surface parasites on or intracellularly in porcine red blood cells (RBC) (Groebel et al., 2009; Hoelzle, 2008). *M. suis* is distributed worldwide and causes serious economic losses to the pig industry (Hoelzle, 2007; Ritzmann et al., 2009). Recent studies have revealed that *M. suis* may also infect humans who have close contact with *M. suis*-infected animals (Yang et al., 2000). In these reports, symptoms including mild pyrexia, haemolytic anemia and icterus have been described, indicating zoonotic potential.

The adhesion of *M. suis* is crucial to its unique RBC-associated 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, the adhesion-mediating proteins and underlying adhesion mechanisms are unknown as yet. Owing to the lack of a culture system, identification of adhesion structures has been difficult. In addition to MSG1 protein, α-enolase is the second adhesion protein of *M. suis*, and may be involved in the adhesion of *M. suis* to porcine red blood cells (RBC) (Hoelzle et al., 2007b; Schreiner et al., 2011).

In the environment of the RBC, direct contact can occur between RBCs and *M. suis*. Thus, peripheral blood mononuclear cells (PBMC) may be used to study the interactions between *M. suis* and host cells. The Y2H system is typically used to find unknown interactive proteins by using a certain protein. This system is an effective and accurate method for researching protein interactions that can be verified in vivo and in vitro. A bait vector, pGBKT7-α-enolase, was constructed in the current study; it expresses the *M. suis* α-enolase protein in the yeast.

The glycolytic α-enolase may be involved in the interaction of *M. suis* with its host cells, because the α-enolase of streptococci and *Aeromonas hydrophila* is known to perform an alternative function as a surface protein that 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 genome of *M. suis* (Oehlerking et al., 2011), the α-enolase gene contains a total of 1623 nucleotides and 540 amino acids. Position 1167 is occupied by adenine, as a result of TGA, which encodes tryptophan in *M. suis*. However, this will lead to the termination of gene translation in the yeast, and therefore use of a primer mutation method is required to replace adenine with guanine and therefore to obtain the full-length α-enolase gene. The PBMC cDNA library was screened using the bait vector in the Y2H system. The identified proteins provide useful information for study of the role of α-enolase in the interaction between the *M. suis* and host cells.

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http://dx.doi.org/10.1016/j.rvsc.2014.07.003
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Table 1
Primers used in this study.

Primer	Sequences (5′→3′)	Length of the PCR products (bp)
P1-F	TTACTGAATGAGTATAGGGAGC	2125
P1-R	ATTCGGGAGATGAAATAAGA	
P2-F	CGC**GGATCC*CCATGGCTTTTAGTATAGAAAATCTT	1192
P2-R	GAGATAATACATGA*C*CATCCAGCTTTCT	
P3-F	AGAAAGCTGGATG*G*TCATGTATTATCTC	480
P3-R	ACGC**GTCGAC*TTAGGATTTTGAAAATCTTGTTT	

* *BamH I* and *Sal I* restriction enzyme sites; *N* mutation position.

2. Materials and methods

2.1. Vector construction

Using the genomic DNA of *M. suis* in Yanbian in Jilin province as a template and P1-F, P1-R as primers (Table 1), reactions were carried out at 94 °C for 5 min for initial denaturation, followed by 30 cycles of 94 °C denaturation for 45 s, 58 °C annealing for 45 s, 72 °C extension for 150 s, and 72 °C for 10 min. The PCR products (10 μl) were analysed by 1% agarose gel electrophoresis, followed by ethidium bromide staining. The product was inserted into the vector pMD18-T, and the plasmid was sent to biotech companies (Invitrogen, China) for sequencing. A sequence including the full length of the α-enolase gene was obtained.

Using the plasmid as a template, two of the fragments were amplified using primers P2-F, P2-R and P3-F, P3-R (Table 1). Finally, primers P2-F and P3-R amplified the full length of the α-enolase gene. The 1623-bp PCR product was inserted into the *BamH I/Sal I* sites of the expression vector pGBKT7. The authenticity and correct orientation of the cloned sequence were confirmed by restriction digestion and sequencing.

2.2. Detection of the activation and toxicity of the bait plasmid

To examine the activation and toxicity of the bait plasmid, competent yeast cells were prepared and transformed with the bait plasmid (pGBKT7-α-enolase), following the methods in the Yeast Protocols Handbook (Clontech Laboratories). At the same time, the empty plasmid pGBKT7-Rec was transformed as the negative control. The transformation competent cells were plated onto tryptophan defective synthesis plates (SD/-Trp) at 30 °C until clones appeared. A single colony was isolated and identified by colony PCR. Afterwards, the single, isolated colony of bait strain Y2H Gold, with bait plasmid (pGBKT7-α-enolase) and an “empty” transcription-activating domain (AD) vector, was plated on the following media: SD/–Trp/–Leu (SD/–2), SD/–Trp/–Leu/–His/–Ade (SD–4), SD/–Trp/–Leu/–His/–Ade/x-a-Gal/Aba (SD/–4/X/A). The plates were incubated at 30 °C for 3–6 days to observe whether the transformant colonies were white or did not grow, thus confirming the activation of the bait protein. The single, isolated colony with plasmids was inoculated into an SD/–Trp medium at 30 °C in a shaker at 250 rpm; 5 ml of medium was collected every 2 h for 38 h. The optical density was detected at a wavelength of 600 nm, and the growth curve of the yeast cells was drawn to determine whether the bait protein was toxic to the transformed cells.

2.3. Generation of a PBMC cDNA library

The PBMC maintained in our laboratory were cultivated to extract total RNA, following the procedures of the user manual of the RNeasy Plus Mini Kit (QIAGEN, Germany). The total RNA extracted was used as a starting material to synthesise first-strand cDNA. Long-distance (LD)-PCR was adopted to synthesise double-stranded cDNA. All procedures were performed according to the user manual of the Make Your Own “Mate & Plate™” LibrarySystem (Clontech, USA). Thus, the double-stranded (ds) cDNA of the PBMC was obtained. Finally, the synthesised dscDNA was purified by a BD CHROMA SPINS TE 400 column to remove cDNA smaller than 200 bp.

2.4. Screening of the PBMC library and selection of M. suis α-enolase interactive clones

Fresh competent yeast cells were prepared using the TE/LiAc method. The LiAc cotransformation method was adopted to facilitate the simultaneous transformation of the bait vector pGBKT7-α-enolase, dscDNA, and linearised DNA-AD vector pGADT7-Rec into the yeast competent cells. The cells were then grown on SD/–Trp/–Leu/–His/–Ade medium for 8–10 days at 30 °C. All the colonies larger than 2 mm on the SD/–Trp/–Leu/–His/–Ade medium were subsequently inoculated onto the SD/–Trp/–Leu/–His/–Ade/X-a-Gal/Aba medium for 3–6 days at 30 °C. Blue colonies were considered to be positive clones.

2.5. Co-transformation of library plasmid to obtained positive clones, and sequencing analysis

Yeast plasmids were extracted from the liquid medium using a yeast plasmid DNA kit (OMEGA, USA). The plasmids from each yeast colony represented a mixture of bait plasmid and at least one type of AD/library plasmid, which needed to be separated in *Escherichia coli*. The isolated plasmids were transformed into DH5α competent cells, which were then grown on an LB medium containing 50 μg/ml ampicillin to identify the AD/library plasmids. Each isolated cDNA fusion plasmid and bait plasmid were co-transformed into yeast competent cells Y2H Gold. Clones were added dropwise to the SD/–Trp/–Leu; SD/–Trp/–Leu/–His/–Ade and SD/–Trp/–Leu/–His/–Ade/X-a-Gal/Aba plates and incubated for 3–6 days at 30 °C to obtain positive clones. The pGBKT7-53 and pGADT7-T, known to interact in the Y2H Gold assays, were used as positive controls, and pGBKT7-Lam and pGADT7-T were used as negative controls. The cDNA insertion was amplified by PCR using universal primers for pGADT7. The plasmids and PCR products were sequenced and compared to proteins in GenBank for similarity.

2.6. Construction of the α-enolase–cellular protein interaction network

An α-enolase–cellular protein interaction network was constructed on the basis of the data on the identified proteins. According to the experimental results, α-enolase is likely to interact with four host cell proteins: beta-actin, 60S ribosomal protein L11, clusterin precursor and endonuclease/reverse, based on the correlation between the proteins in the STRING 9.0 database (Szklarczyk et al., 2011).

3. Results

3.1. Construction and identification of the bait vector

A sequence including the full length of the α-enolase gene was successfully amplified by PCR from the *M. suis* genome (Fig. 1a). The gene encoding α-enolase was obtained by the method of mutation and gene spliced from the plasmid, giving the full-length α-enolase gene (Fig. 1b). The products were identified by nucleotide electrophoresis, purified, and subsequently inserted into plasmid pGBKT7. The recombinant plasmid, pGBKT7-α-enolase, was confirmed to be correct using PCR and double restriction enzyme digestion by *BamHI/SalI* (Fig. 1c). Sequence analysis revealed that the

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