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Mycoplasma pneumoniae Protein-Protein Etkileşimlerinin Maya İkili-Hibrid Yöntemi ile Analizi

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ÖZ

Protein etkileşimlerini tanımlamak, protein fonksiyonlarını belirlemede önemli bir adımdır. Bilinmeyen bir proteinin fonksiyonu, bu nedenle, anotasyonlu etkileşim partnerlerinin tanımlanmasından çıkarılabilir. Tipik olarak, maya ikili-hibrid sistemi (Y2H), protein-protein etkileşimlerini tespit etmek için en yaygın kullanılan genetik testtir. Mikoplazmalar, genomu tamamen dizilen ilk organizmalardan biridir ve minimal hücreler ile sentetik biyolojiyi çalışmak için ilginç bir modeldir. Ancak mikoplazmalarda protein-protein etkileşimlerini incelemek için kullanılan başarılı bir Y2H sistemi bulunmamaktadır. Bu çalışmada, insan patojenik bakterisi *Mycoplasma pneumoniae* kullanarak mikoplazmalar için kullanılabilir başarılı bir Y2H sistemi geliştirme ve uygulama hedeflenmiştir. İlk denemelerde, *M. pneumoniae*'nin hareket mekanizması ve hücre iskeleti bütünlüğünü korumada görevli olduğu bilinen proteinler olan P1 adhezin (MPN141), HMW1 (MPN447) ve HMW2 (MPN310) arasındaki etkileşimlere odaklanılmıştır. Başarılı bir Y2H sistemi geliştirmek için önemli iki ana unsurun olduğu bulunmuştur. İlk unsur, türler arası kodon kullanım farklılıklarına dikkat ederek kodon uyumluluğunu sağlamasıdır. UGA kodonu prokaryotlar ve ökaryotlar boyunca bir durdurma kodonu olarak kodlandığı yerde mikoplazmalarda triptofan amino asidi olarak kodlanır. İlgili hedef gen dizilerinde UGA kodonu UGG olarak düzenlenmiştir. İkinci unsur ise etkileşimleri incelenecek proteinlerin ikincil yapıları dikkate alınarak Y2H vektörlerinin tasarlanmasıdır. P1 adhezin, HMW1 ve HMW2 proteinlerinin ikincil yapıları incelenerek, Y2H vektörleri tasarlanırken hedef proteinlerin transmembran segmenti içermemeleri ve kıvrımlı-bobin, α-sarmal ve β-levhaların bozulmayacak şekilde fragmentlere ayrılmasına dikkat edilmiştir. Sonuçlarımız, P1 adhezin en uç C-terminal bölgesi ile HMW1 ve HMW2 proteinlerinin C-terminal bölgeleri arasında etkileşim olduğunu göstermiştir. Bu çalışma, *M. pneumoniae*'de Y2H'nin ilk başarılı denemesi olup, diğer mikoplazma türlerinde yapılacak denemeler için literatüre katkı sağlayacağı düşünülmektedir.

Analysis of *Mycoplasma pneumoniae* Protein-Protein Interactions by Yeast Two-Hybrid Method

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ABSTRACT

Defining protein-protein interactions is a crucial step in elucidating protein functions. Consequently, the function of an uncharacterized protein can be determined by recognizing its known interaction partners. The yeast two-hybrid (Y2H) system is commonly regarded as the most popular genetic assay for identifying protein-protein interactions. *Mycoplasmas* are among the first organisms with fully sequenced genomes and serve as intriguing models for studying minimal cells and synthetic biology. However, a successful Y2H system has not yet been established to examine protein-protein interactions in mycoplasmas. In this study, we aimed to develop and implement a viable Y2H system for mycoplasmas using the human pathogenic bacterium *Mycoplasma*

pneumoniae. The initial trials concentrated on examining the interactions among proteins that are recognized for their roles in the motility of *M. pneumoniae* and the preservation of cytoskeletal integrity, particularly the P1 adhesin (MPN141), HMW1 (MPN447), and HMW2 (MPN310). Our findings indicate that two primary factors are essential for successfully developing of a Y2H system. The first factor pertains to ensuring codon compatibility by considering differences in interspecies codon usage. While the UGA codon encodes a stop codon across prokaryotes and eukaryotes, in mycoplasmas, it is translated as tryptophan. Thus, the UGA codon in the relevant target gene sequences has been modified to UGG. The second factor involves designing Y2H vectors by considering the secondary structures of the proteins to be examined for interactions. By analyzing the secondary structures of P1 adhesin, HMW1, and HMW2 proteins, it was ensured that the target proteins do not contain transmembrane segments and that the structures of the coiled-coil, α-helix, and β- sheet were preserved when fragmented. Our results demonstrate an interaction between the extreme C-terminal region of P1 adhesin and the C-terminal regions of HMW1 and HMW2 proteins. This study represents the first successful attempt at Y2H in *M. pneumoniae*, and it is anticipated that it will contribute to the literature for further experiments on other mycoplasmal species.

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1. Introduction

Protein-protein interactions (PPIs) are fundamental biological phenomena that play a crucial role in virtually every cellular process. These interactions involve the physical binding of two or more proteins, leading to a range of functional outcomes essential for maintaining cellular homeostasis, signalling, and regulation. PPIs are involved in critical processes such as enzyme activity modulation, signal transduction, immune responses, and cellular structural integrity (Kuzmanov and Emili, 2013). The combination of experimental and computational methods provides a comprehensive framework for identifying and characterizing these interactions, ultimately contributing to a deeper understanding of cellular functions (Ori et al., 2016; Titeca et al., 2018). PPI studies employ various methods to identify and characterize how proteins interact with each other in biological systems. Historically, the study of PPIs has evolved from traditional biochemical methods, such as yeast two-hybrid assays and co-immunoprecipitation (Co-IP), to advanced high-throughput techniques like mass spectrometry and protein microarrays. Recent advances in computational biology and bioinformatics have further enhanced our understanding of PPIs by facilitating the prediction and modeling of interactions based on protein structural data and sequence homology (Hashemifar et al., 2018; Zhang et al., 2020). Machine learning algorithms and network analysis techniques have also emerged as powerful tools for dissecting complex PPI networks, enabling researchers to predict the functional consequences of specific interactions and identify critical nodes within signaling pathways (Sun et al., 2017; Jumper et al., 2021). Despite these advancements, many challenges remain in the study of PPIs. The transient and context-dependent nature of many interactions complicates their detection and interpretation. The yeast two-hybrid (Y2H) system is a commonly used experimental method for investigating protein- protein interactions *in vivo* using yeast cells. The underlying principle of the Y2H system is based on the

modular structure of transcription factors, which typically consist of two domains: a DNA-binding domain (DBD) and a transcriptional activation domain (AD). In this system, a protein of interest known as the "bait" is combined with the DBD, whereas a second protein referred to as the "prey" is linked to the AD. When both proteins are expressed in yeast cells, interaction between the bait and prey brings the DBD and AD together, resulting in the activation of a reporter gene. This activation allows for the growth of yeast on selective media (his3 which allows yeast to grow in selective media) or measurable enzymatic activity or (lacZ which can produce a color change), indicating a successful interaction (Makuch, 2014; Mehla et al., 2015). *Mycoplasmas* are one of the first organisms to have their genomes fully sequenced and an intriguing model for studying minimal cells and synthetic biology. *Mycoplasmas* typically have extremely small genomes, often fewer than 1,000 genes (Razin et al., 1998). This can make them a potential model organism to study genetic function and gene interactions, as there are fewer genes to analyze and manipulate. But, studying PPI in *Mycoplasmas* is challenging due to a modification in their genetic code. They use UGA as tryptophan which is a stop codon throughout prokaryotes and eukaryotes (Inamine et al., 1990). Moreover, the expression of heterologous genes introduced into mycoplasmas can be unpredictable due to differences in regulatory elements, which may lead to low or variable protein production (Ana et al., 2024). These challenges necessitate the development and optimization of techniques and methods tailored to mycoplasma PPI research to achieve successful functional studies. Recent advances in proteomics and molecular biology have enabled a deeper understanding of the interactions between mycoplasma proteins and host cells. Techniques such as co-immunoprecipitation, mass spectrometry, and fluorescence microscopy have been pivotal in elucidating these complex protein-protein interactions (Wang et al., 2024). Studies have also revealed that certain mycoplasmas can translocate into host cells, where they can manipulate host cellular machinery and signaling pathways, thereby disrupting normal cellular functions (Liu et al., 2022). Despite the advances made in this field, many aspects of mycoplasma protein interactions remain poorly understood, particularly concerning the full repertoire of mycoplasmal effectors and their mechanisms of action. This lack of comprehensive understanding hinders the development of effective therapeutic interventions and vaccines against mycoplasma infections. *Mycoplasma pneumoniae* is a bacterium belonging to *Mycoplasmas* family causes respiratory infections, notably atypical pneumonia, particularly in children and young adults. *M. pneumoniae* possesses unique characteristics, one of which is its gliding motility which plays a significant role in its pathogenicity (Bajanti et al., 2018; Torres et al., 2021). Gliding motility aids in the adhesion of *M. pneumoniae* to respiratory epithelial cells. By moving along the surface, it can find and tightly bind to host cells, increasing its ability to colonize the respiratory tract. The ability to adhere to the respiratory epithelium is critical for infection. *M. pneumoniae* uses surface proteins (adhesins) to attach to host cells. Unlike more conventional bacterial motility, which often relies on flagella or cilia (Basemann et al., 1982). The gliding motility of *M. pneumoniae* is a sophisticated mechanism that enables it to move along surfaces through a combination of adhesion and internal cellular dynamics. The gliding machinery is composed of a network of proteins

including nap-like surface protrusions (P1 adhesin complex including the P40/P90 polypeptides) and internal structure which consists of a terminal button, paired plates (HMW1 and HMW2) and a bowl complex (Hegermann et al., 2002) (Figure 1A-B-C). P1 adhesin (MPN141) is a protein consisting of 1,627 amino acids, known to be the main adhesin that enables bacteria to attach to host cells (Feldner et al., 1982; Hu et al., 1982; Inamine 1988). It is primarily concentrated at the tip of *M. pneumoniae* but is also found distributed across the surface of the mycoplasma (Baseman et al., 1982). The high-molecular-weight proteins (HMW proteins) are components of a cytoskeleton-like structure in *M. pneumoniae*, and their activities imply that they might have a scaffolding function (Krause, 1996; Hahn et al., 1998; Willby et al., 2002).

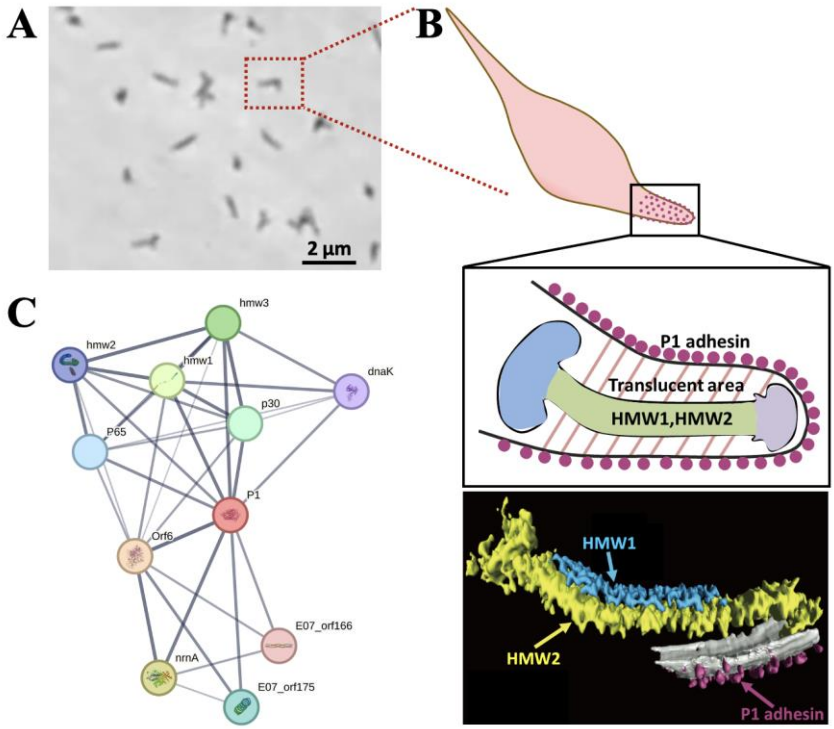

Figure 1. Gliding mechanism of *Mycoplasma pneumoniae*. **A)** Phase contrast microscopy image of *M. pneumoniae* (scale bar: 2 µm). **B)** Schematic of gliding machinery and the localization important proteins. **C)** The network of interactions among P1 adhesin and related proteins. The protein interaction network was constructed using the STRING 9.0 database.

HMW1 (MPN447) is a 1018 amino acids long membrane protein located on the surface of the mycoplasma especially in the attachment organelle and paired plates (Stevens and Krause, 1991). HMW2 (MPN310) is an 1818 amino acids long protein predicted to form dimeric coiled coils and is essential for the development of a cytoskeleton-like structure as well as for cytadherence (Balish et al., 2003). Over the last three decades, increasing evidence suggests that *M. pneumoniae* has a cytoskeleton-like structure, likely serving as a replacement for its absent cell wall and may provide the essential support needed to maintain and stabilize the cell integrity of *M. pneumoniae* and facilitate gliding motion (Radestock and Bredt, 1977; Meng and Pfister, 1980; Krause, 1996). Numerous studies (Krause et al.,

1997; Romero-Arroyo et al., 1999) have demonstrated the connection between the positioning of the attachment organelle and cytadherence. Researchers isolated spontaneous mutants that exhibited a cytadherence-negative phenotype; these mutants contained the P1 adhesin but lacked HMW1 and HMW2 proteins (Krause et al., 1982; Layh-Schmitt et al., 1995; Popham et al., 1997; Hahn et al., 1998; Bose et al., 2009).

M. pneumoniae is distinguished from other bacteria by its minimalistic genome and lack of a cell wall, affording it unique adherence capabilities and pathogenic mechanisms. The interactions of *M. pneumoniae* with host cells are orchestrated through a network of proteins that facilitate adherence, immune evasion, and manipulation of host cellular processes. To dissect these complex interactions, co-immunoprecipitation (Co-IP) has emerged as a critical analytical tool, enabling the identification of protein complexes and interactions in biological systems (Wang et al., 2024). Recent advances in mass spectrometry combined with Co-IP have enhanced the resolution of protein interactions for other mycoplasmas, offering a deeper insight into the molecular basis of mycoplasmal pathogenesis (Yu et al., 2019). While considerable progress has been made utilizing Co-IP to uncover crucial protein interactions, many aspects of the *M. pneumoniae*- host interface remain understudied. The identification of novel proteins and their interaction networks is essential for a comprehensive understanding of the pathogenicity of this organism. In this study, we aimed to develop a Y2H system for *M. pneumoniae* to easily examine possible interactions between motility and cytoskeletal related proteins. For this purpose, we focused on P1 adhesin protein, along with the HMW1 and HMW2 proteins, which are known to be involved in motility and the cellular cytoskeleton and have established interactions; however, detailed interaction studies have yet to be conducted. Key considerations for developing a successful Y2H system included ensuring codon compatibility by attending to interspecies codon usage differences, as well as designing Y2H constructs to avoid structural errors in the corresponding target fragments.

2. Material and Methods

2.1. Strains and Cultivation

Mycoplasma pneumoniae M129 (ATCC29342) was grown in SP4 medium at 37°C in tissue culture flasks (NEST, China), as described previously (Tully, 1983). *Saccharomyces cerevisiae* strain AH109 was grown according to the Yeast Protocol Handbook (Clontech Laboratories, USA) in YPDA medium (Yeast Extract 1%, Bacto Peptone 2%, Glucose 2%, Adenine 0.02%). The *Escherichia coli* strain, stellar cells from In- fusion PCR EcoDry Cloning Kit (TaKaRa Bio, Japan) was grown in LB medium.

2.2. Plasmid Construction

All routine DNA manipulation techniques, digestion with restriction endonucleases and transformation of *E. coli* were performed according to the Cold Spring Harbor Laboratory Manual (Sambrook et al., 1998). The pGADT7 and pGBKT7 vectors were obtained from Takara Bio, USA. The primers were synthesized by oligonucleotide synthesizing service of Macrogen (Macrogen, Korea), and are listed in

(Table 1). *Mycoplasma pneumoniae* 129 genomic DNA was prepared using the Genomic-tip System (Qiagen, Hilden, Germany). P1 Domain-III, HMW1 and HMW2 fragments were amplified by conventional PCR from isolated genomic DNA using KOD-FX-Neo DNA Polymerase (Toyobo, Japan). The fragmentation pattern was designed to avoid frameshift in yeast two-hybrid (Y2H) vector. All primers used for In-fusion cloning consisted of gene-specific sequences and a 15bp overlapping sequence. PCR reactions were performed with KOD-FX-Neo DNA Polymerase (Toyobo, Japan). The PCR protocol was 5 min at 95°C for initial denaturation, followed by 30 cycles of 95°C denaturation for 1 min, 45°C annealing for 30 s, 68°C extension for 1 min, and 68°C for 4 min. 5 µl of the PCR products were analyzed by 1% agarose gel electrophoresis, followed by GelRed staining (Biotium, USA). To obtain vectors suitable for the In-Fusion reaction, pGADT7 and pGBKT7 were linearized with *Eco*RI (NEB, USA). All cloning reactions were performed by using In-Fusion HD EcoDry PCR Cloning Kit following the manufacturer's protocol (Takara Bio, Japan). Briefly, 1 µl of *Eco*RI digested vector and 3 µl of PCR product were mixed with In-Fusion HD EcoDry pellet and incubated 15 min at 37°C, followed by 15 min at 50°C. Then, 2.5 µl of the reaction mixture was transformed into *E. coli* stellar competent cells provided with In-Fusion HD EcoDry PCR Cloning Kit. The transformant was grown on LB solid medium with an appropriate antibiotic (ampicillin for pGADT7- 100 µg/ml) and kanamycin for pGBKT7- 50 µg/ml) for 12–16h at 37°C. HMW1 and HMW2 fragments were inserted into pGADT7 as prey vectors.

The resulting HMW1 prey plasmid were named as hmw1/1-1, hmw1/2-1, hmw1/2-2, hmw1/3-1, hmw1/3-2, and hmw1/3-3. The resulting HMW2 prey plasmids hmw2/1-1, hmw2/1-2, hmw2/1-3, hmw2/1-4, hmw2/1-5, hmw2/1-6, and hmw2/1-7. P1 Domain-III was inserted into pGBKT7 as bait vector resulting in pGBKT7-P1-III. The stop codons (TGA) in HMW1 and HMW2 fragments were changed to tryptophan (TGG) by point mutation using specific primers Table 1. Site-directed mutagenesis was performed by KOD-Plus- Mutagenesis kit according to the manufacturer's protocol (Toyobo, Japan). The presence of correct fragments was confirmed by Sanger sequencing using the T7 universal sequencing primer (ABI Hitachi, Applied Biosystems 3130 Genetic Analyzer).

Table 1. Primers used in this study.

Primer	Sequences (5′→3′)
P1-F	CACACGAATTCCCGATGCATAAAAATAAACAGGCA
P1-R	CACACGGATCCTTATGCCGGTTTTTTCGGAGGCTG
hmw1/1-1-F	CACACGAATTCATGAAGAAAAGCAAAGAAGCAGTT
hmw1/1-1-R	CACACGGATCCATCTGCTTCCAAACCAAAGTCTTC
hmw1/2-1-F	CACACGAATTCGTACCAGCACCAGAAGTTGCTGAA
hmw1/2-1-R	CACACGGATCCCTCTGTAACTACCTGAGGTGTAAT
hmw1/2-2-F	CACACGAATTCCCTGTAGTAGCTGTTGTAGAACAT
hmw1/2-2-R	CACACGGATCCAACGGGTGCTACTGCTGGTTGAGG
hmw1/3-1-F	CACACGAATTCGCTGATATGGATTATGTCTTACAT
hmw1/3-1-R	CACACGGATCCCCATACAACCTCACTGTTACTGTG
hmw1/3-2-F	CACACGAATTCCGAGTAAGCGAACCGAAAACAGTT
hmw1/3-2-R	CACACGGATCCTTGGTACACCGAAAGGTCTTTAGC
hmw1/3-3-F	CACACGAATTCAAGGATGAACTGCTACGCTCTTTA
hmw1/3-3-R	CACACGGATCCCCAATAATCAAGATTAAAATCCCC
hmw2/1-F	CACACGAATTCATGAATGATACTGACAAGAAGTTC
hmw2/1-R	CACACGGATCCCACCTTAAGTGTCGATTCTTCATC
hmw2/2-F	CACACGAATTCGTTTCTAAAGAACAAGCTCAAGAC
hmw2/2-R	CACACGGATCCCCAATCCTCTGCAATCTTTTGTGC
hmw2/3-F	CACACGAATTCGCACATCTCAAACAAAACAAACAC
hmw2/3-R	CACACGGATCCACTGCGTTCGCGCTCTAATAATTC
hmw2/4-F	CACACGAATTCTTTTTACTGCAAAAACAACGTGAA
hmw2/4-R	CACACGGATCCAGCAAAATCAAGCGTTAGTTGGTT
hmw2/5-F	CACACGAATTCTTACTACGTAAAGTGCAACACAAT
hmw2/5-R	CACACGGATCCAGCTTCATTTTGGGCTTTTAGCTC
hmw2/6-F	CACACGAATTCACGGCACACAAAAATCGTGAAGTT
hmw2/6-R	CACACGGATCCCCAAGGATATGGGTAAGGATAGTT
hmw2/7-F	CACACGAATTCTTTTATCCGCAACAAAAACAAGAA
hmw2/7-R	CACACGGATCCCCATTTAGCTGCTTTTTGGGCAAT
hmw1-sd-F	AAGTAACCAGACCAAACCCACTCC
hmw1-sd-R	GGAGTGGGTTTGGTCTGGTTACTT
hmw2-sd-F	TGAAAGTCAGCCCAATTGGCATAA
hmw2-sd-R	TTATGCCAATTGGGCTGACTTTCA

2.3. Yeast Two- Hybrid Assay

Prey and bait vectors were co-transformed into the yeast strain AH109 by LiAc/SS Carrier DNA/PEG method (Gietz and Woods, 2006). The autoactivation of the reporter genes in yeast strain AH109 of the bait vector, pGBKT7-P1-III was examined in the absence of a prey protein, pGADT7-empty. Transformants were plated on a minimal synthetically defined (SD) medium without Trp (SD/-Trp). pGBKT7-empty vector was used as a negative control.

2.4. Screening of Interactions by Dropwise Test

The positive clones expressing bait protein P1 Domain-III interacting with prey proteins were selected on SD/-Leu/-Trp medium at 30°C for 5 days. For further selection, the positive clones were screened on SD/- His/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp. To limit background growth and detect the strength of the interaction between bait and prey, screening was performed with SD/-His/-Leu/-Trp plates that are supplemented with 1mM and 3mM of 3-Amino-1,2,4-Triazol (3-AT) a competitive inhibitor of the *his3* reporter gene. The resulting colonies were selected and identified by colony PCR.

2.5. Construction of P1 Adhesin-Cytoskeletal Protein Interaction Network

The protein interaction network was constructed on the basis of the data on the identified proteins. Accord- ing to the experimental results, P1 adhesin is likely to interact with two cytoskeletal proteins: HMW1 and HMW2, based on the correlation between the proteins in the STRING 9.0 database (Szklarczyk et al., 2011).

3. Results and Discussion

Considering previous studies on interaction investigations, the 81-amino acid region following the transmembrane (TM) domain at the C-terminus of the P1 adhesin protein (P1-Domain-III) was selected as the primary target interaction partner. To gain a detailed understanding of which regions of the HMW1 and HMW2 proteins interact with P1-Domain-III, HMW1 and HMW2 proteins were grouped into smaller fragments (Figure 2A-B-C). Structurally, the HMW1 protein consists of region 1, region 2, and region 3. The hmw1 gene was analyzed to determine the boundaries of these regions and to create the yeast two-hybrid (Y2H) construct fragments. Region 1 was defined to produce the hmw1-1 fragment, measuring 510 base pairs. Region 2 was divided to generate hmw1/2-1 and hmw1/2-2 fragments, sized 501 bp and 555 bp, respectively. Upon examining the secondary structure of region 3, two coiled-coil regions were identified. Without disrupting the structure of these coiled-coil regions, region 3 was split into three fragments: hmw1/3-1, consisting of 330 bp; hmw1/3- 2, consisting of 561 bp; and hmw1/3-3, consisting of 600 bp (Figure 2B).

The same strategy was applied to the HMW2 protein. Upon examining the secondary structure of HMW2, it was observed to contain 11 coiled-coil regions. To maintain the integrity of these coiled-coil structures, HMW2 was divided into seven fragments: hmw2/1-1, consisting of 939 bp; hmw2/1-2, consisting of 753 bp; hmw2/1-3, consisting of 894 bp; hmw2/1-4, consisting of 699 bp; hmw2/1-5, consisting of 933 bp; hmw2/1- 6, consisting of 636 bp; and hmw2/1-7, consisting of 573 bp (Figure 2C). The identified fragments were amplified from the genomic DNA of *Mycoplasma pneumoniae* using PCR with specific primers (Table 1). P1-Domain-III fragment was inserted into pGBKT7 bait vector with the GAL4 DNA binding domain (BD) by In-Fusion cloning resulting pGBKT7- P1-Domain-III. Individual HMW1 and HMW2 fragments were inserted into pGADT7 prey vector (Figure 3).

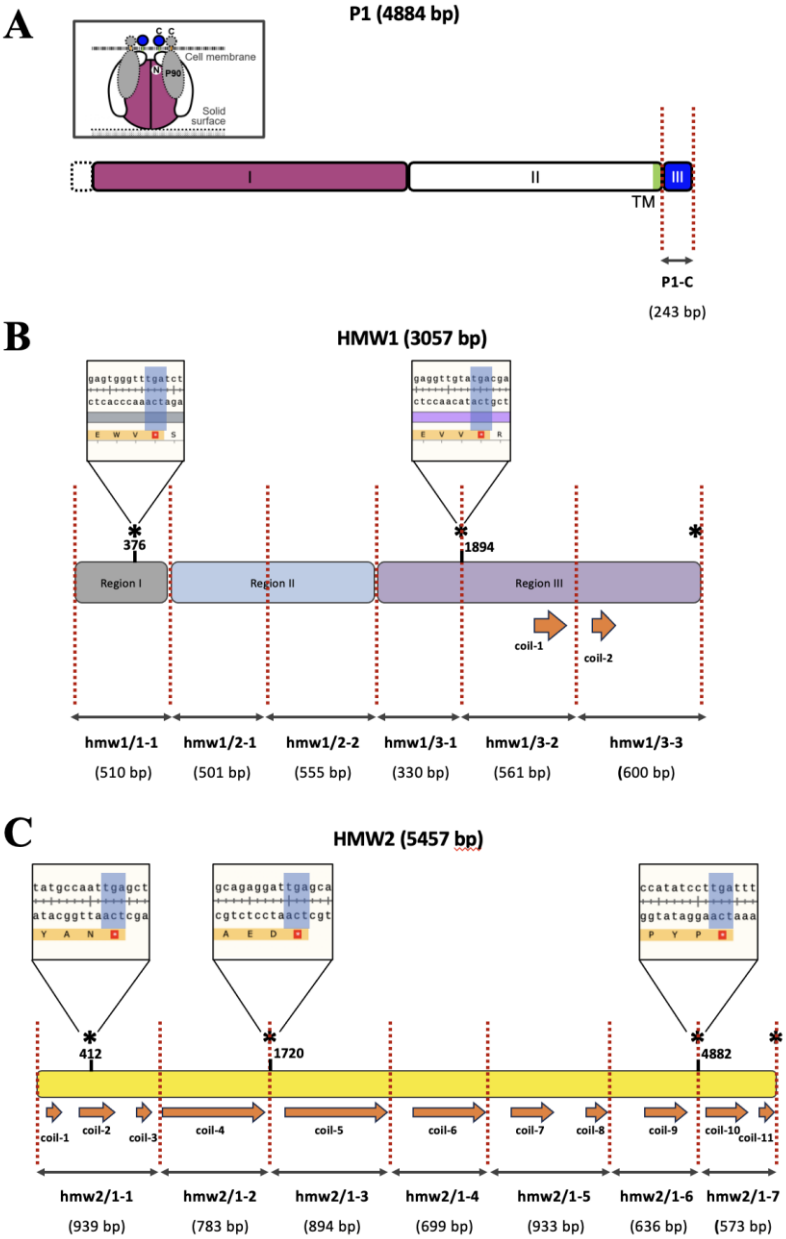

Figure 2. Fragmentation schema and stop codon analyses of P1 adhesin, HMW1 and HMW2 proteins.

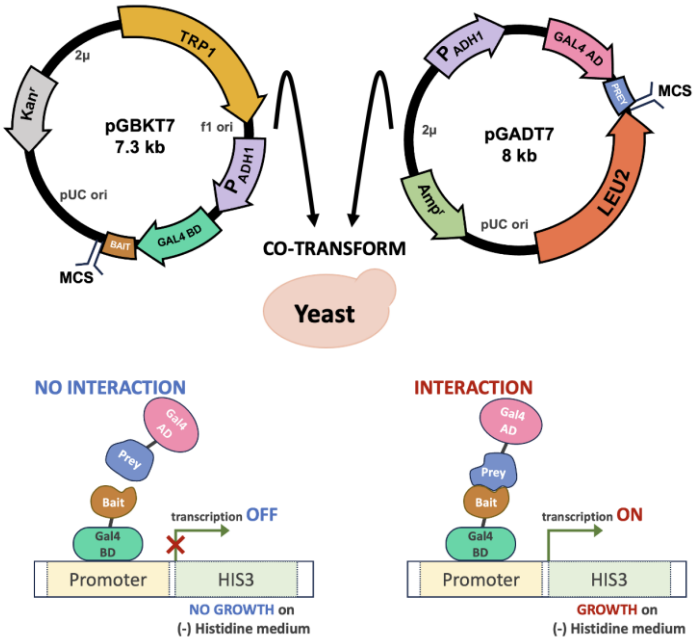

Figure 3. Mechanism of yeast two-hybrid system and plasmids that are used.

In *Mycoplasmas*, the UGA codon is encoded as tryptophan, whereas it is a stop codon in prokaryotes and eukaryotes. So, we investigated whether UGA codons were present in the sequences encoding the relevant proteins. In the *hmw1* gene, two UGA codons were found at positions 376 and 1894 (Figure 2B). In the *hmw2* gene, three UGA codons were identified at positions 412, 1720, and 4882 (Figure 2C). To prevent these codons from being expressed as stop codons in yeast, modifications were made to change them to UGG codons using specific primers by site-directed mutagenesis (Table 1). Correct clones were verified by DNA sequencing. The autoactivation of *his3* reporter gene in the bait (P1-Domain-III) was examined by co-transforming with the empty prey vector. AH109 cells were transformed with bait plasmid pGBKT7-P1-Domain-III and plated on SD/- Trp agar plates and incubated at 30◦C for 2–4 days. pGBKT7-empty vector was used as the negative control. Self-activation assay showed that Y2H transformed with pGBKT7- P1-Domain-III could grow normally on the SD/–Leu/ –Trp plate (Figure 4); it did not grow on the SD/–Trp/–Leu/–His/–Ade. Hence, empty vectors did not activate reporter genes. For the pairwise tests, the yeast two-hybrid strain A109 was co-transformed with plasmids that encode bait and prey fusions of the respective proteins. The interactions between these fusion proteins were assessed by culturing on SD/-His/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp. Furthermore, SD/-His/-Leu/-Trp plates that are supplemented with 1mM and 3mM of 3-AT were used for the screening of activation of HIS3 reporter gene. All possible pairwise interactions among the selected proteins were examined, and these interactions were also tested for nonspecific binding with control plasmids (Figure 5).

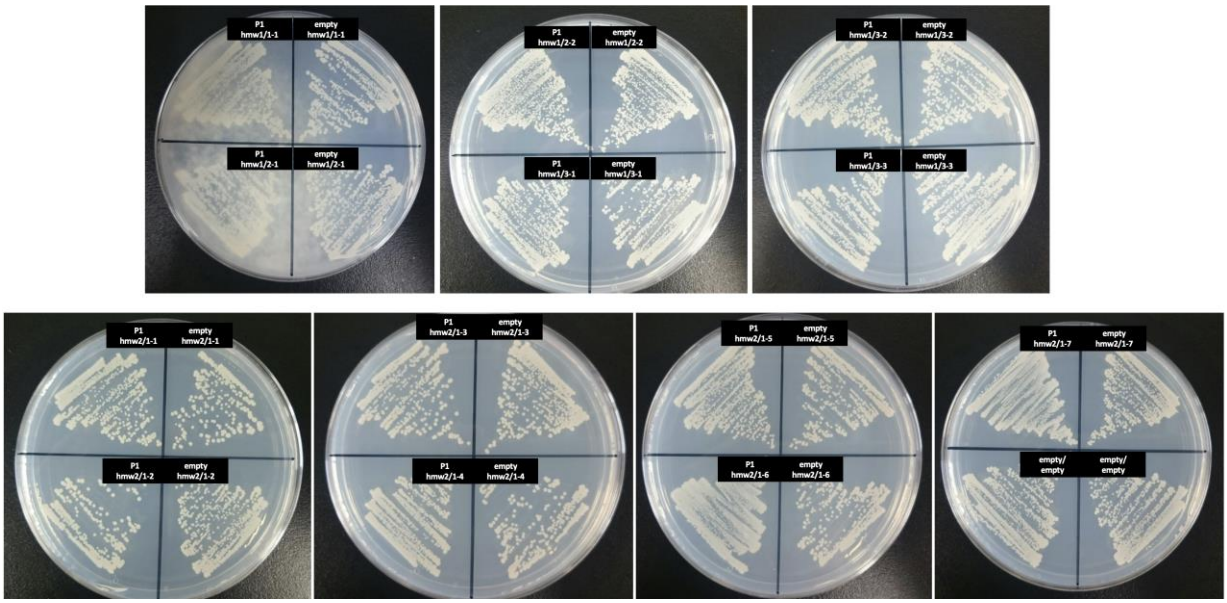

Figure 4. Transformation control of yeast two-hybrid system. The clones grew normally on SD/-Leu/-Trp plates.

Following the Y2H screening, a total of 105 yeast colonies were chosen from SD/-Trp/-Leu/-Ade/-His plates for the determination of positive clones that demonstrated interactions between the bait and prey proteins. According to our results, the P1-Domain-III region shows strong interaction with the hmw1/3-2 and hmw1/3-3 fragments, which correspond to the C-terminal end of the HMW1 protein. These fragments represent the region-3 area located at the C-terminal end of the HMW1 protein. The P1-Domain-III region shows strong interaction with the hmw2/coil6 and hmw2/coil7 fragments which also correspond to the C- terminal end of the HMW2 protein.

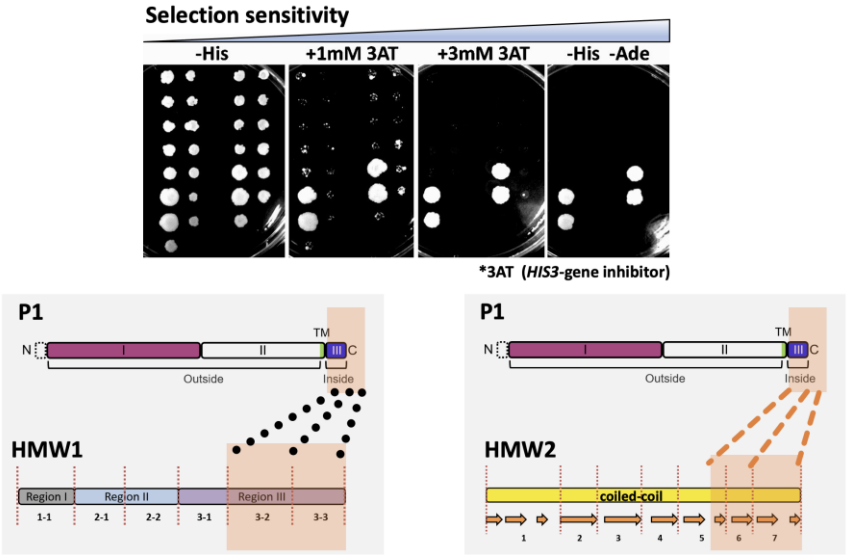

Figure 5. Detection of interaction and activation of reporter gene *his3*. The clones were selected on SD/-His, SD/-His/-Ade, SD/-His/-Leu/-Trp (+1mM 3AT), and SD/-His/-Leu/-Trp (+3mM 3AT) plates. The interactions were mapped on protein schema.

Previous research has thoroughly characterized the regions in P1 (Schurwanz et al., 2009). It is hypothesized that the binding site for sialylated oligosaccharides might be located in the N-terminal region of the protein. This is based on the idea that the amino acid sequences of binding sites are not likely to change quickly, while the C-terminal end is anticipated to be more tucked within the protein's structure. Movements initiated in other regions might be conveyed to the P1 adhesin complex, where the C-terminal end, located internally, could be crucial for transferring force from another internal section of the organelle.

4. Conclusions

Protein interaction networks are central to the biology and pathogenesis of *Mycoplasma pneumoniae*. By elucidating these networks, we can gain valuable insights into the mechanisms of infection, immune evasion, and metabolic adaptation. In this study, we successfully designed a usable yeast two-hybrid system (Y2H) for *Mycoplasma pneumoniae* for the first time. We demonstrated that when designing yeast two-hybrid vectors for *Mycoplasmas*, it is essential to control the codon usage of the proteins to be studied and to consider the secondary structures. Using the newly designed Y2H vectors, we tried to clarify the interaction regions between three important proteins involved in cytoskeletal structure and motility, namely P1 adhesin, HMW1, and HMW2. Our results will not only make essential contributions to the *Mycoplasma* studies, but also will have significant implications for developing novel therapeutics and vaccines. As experimental and computational techniques continue to advance, our understanding of *M. pneumoniae* PPIs will deepen, paving the way for innovative strategies to combat this important pathogen. Future research should focus on the characterization of lesser-known mycoplasma species and their proteins, as well as the implications of these interactions in chronic diseases.

Conflict of interests

Author declare that she has no conflict of interest.

Consent for publication

The author declares that she has contributed 100% to the article.

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