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Novel protein interactions with an actin homolog (MreB) of *Helicobacter pylori* determined by bacterial two-hybrid system

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ABSTRACT

The bacterium *Helicobacter pylori* infects more than 50% of the world population and causes several gastroduodenal diseases, including gastric cancer. Nevertheless, we still need to explore some protein interactions that may be involved in pathogenesis. MreB, an actin homolog, showed some special characteristics in previous studies, indicating that it could have different functions. Protein functions could be realized via protein-protein interactions. In the present study, the MreB protein from *H. pylori* 26695 fused with two tags 10 × His and GST in tandem was overexpressed and purified from *Escherchia coli*. The purified recombinant protein was used to perform a pull-down assay with *H. pylori* 26695 cell lysate. The pulled-down proteins were identified by mass spectrometry (MALDI-TOF), in which the known important proteins related to morphogenesis were absent but several proteins related to pathogenesis process were observed. The bacterial two-hybrid system was further used to evaluate the protein interactions and showed that new interactions of MreB respectively with VacA, UreB, HydB, HylB and AddA were confirmed but the interaction MreB-MreC was not validated. These results indicated that the protein MreB in *H. pylori* has a distinct interactome, does not participate in cell morphogenesis via MreB-MreC but could be related to pathogenesis.

1. Introduction

The bacterium *Helicobacter pylori* infects over 50% of the worldwide population, causing chronic gastritis, and peptic and duodenal ulcers (Eppinger et al., 2004). It is a carcinogen according to the International Agency for Research on Cancer of the World Health Organization (Das and Paul, 2007).

In the course of infection, *H. pylori* uses urease, an essential enzyme for colonization, to catalyze the conversion of urea into carbon dioxide and ammonia for acid resistance in human stomach. *H. pylori* also secretes two major toxins, VacA (vacuolating cytotoxin) and CagA (cytotoxin-associated gene A), into gastric epithelial cells. VacA induces the formation of acid vacuoles and affects mitochondrial function in human cells. CagA is an effector related to the formation of tumors and causes the rearrangement of host cell actin filaments (Monack et al., 2004).

In eukaryotes, the cytoskeleton system is composed of three main kinds of filaments called microtubules, actin filaments and intermediate filaments (Alberts et al., 2008). It was previously considered that prokaryotes lacked a cytoskeleton; however, the existence of a cytoskeleton in prokaryotes was reported in 1992 (Bork et al., 1992; RayChaudhuri and Park, 1992; Mukherjee et al., 1993). *H. pylori* is one of a few bacteria that were confirmed containing all of the three kinds of filaments.

Of the bacterial cytoskeleton elements, the protein homologous to eukaryotic actin was called MreB. It has similar tridimensional structure to eukaryotic actin and can assemble to form filaments in the presence of ATP or GTP *in vitro* or *in vivo*. This protein showed several functions such as participation in cell viability (Bendezu et al., 2009), cell division (Jones et al., 2001), chromosome segregation (Kruse and Gerdes, 2005), cell shape and morphogenesis (Figge et al., 2004; Takacs et al., 2010) as well as the location of the chromosomal

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replication machinery (Soufo and Graumann, 2005). In most rod-shaped bacteria such as *E. coli* and *Bacillus subtilis*, the *mreB* deletion affects the cell shape and is lethal to cells (Wachi and Matsuhashi, 1989; Varley and Stewart, 1992; Jones et al., 2001). However, the *mreB* deletion in *H. pylori* does not affect neither the shape nor the viability of the bacterium but causes a decrease in the urease activity (Waidner et al., 2009). In addition, MreB was found as an antigen in patients with gastric cancer (Lin et al., 2006). Such information suggests that MreB may be associated with pathogenesis in *H. pylori*.

MreB-associated proteins have been analyzed only in *B. subtilis* (Kawai et al., 2011) using pull-down assay and in *E. coli* using both pull-down assay (Arifuzzaman et al., 2006) and tandem affinity purification (TAP) (Butland et al., 2005). Many proteins that interact directly with MreB were confirmed via bacterial or yeast two-hybrid systems, or by co-immunoprecipitation. In general, MreB partner proteins are principally related to the maintenance of cell shape, such as MreC, RodZ, FtsZ, RodA, PBPs, MurF, MraY, and MurG (Defeu Soufo and Graumann, 2006; Bendezu et al., 2009; Kawai et al., 2009; van den Ent et al., 2010; White et al., 2010; Dominguez-Escobar et al., 2011; Garner et al., 2011; Tan et al., 2011; Fenton and Gerdes, 2013). Some other partners in bacteria were also reported (Espeli et al., 2003; Madabhushi and Marians, 2009; Munoz-Espin et al., 2009; Mauriello et al., 2010; Ouellette et al., 2014). Based on the characteristics of *H. pylori* MreB (Lin et al., 2006; Waidner et al., 2009), it is needed to study the functions of this protein through identification of proteins interacting with MreB. In the present study, the possible interaction proteins with MreB in complexes were firstly obtained by pull-down assay and then were further evaluated by bacterial two-hybrid system (BACTH). Several new interactions were demonstrated, indicating that MreB in *H. pylori* could participate in pathogenesis.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli DH5α strain grew in LB with 100 μg/ml ampicillin. *E. coli Codon Plus* strain grew in 2 × YT medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The *H. pylori* 26695 cells grew on Casman agar base (DIFCO) supplemented with 10% defibrinated horse serum and incubated at 37 °C for 72 h under microaerophilic conditions.

2.2. Construction of recombinant protein MreB and its control for pull-down assay

To amplify the *mreB* gene (HP1373), the primers MreBF and MreBR were used for PCR (Table S1). Each primer contains a restriction site, *Nde*I for MreBF and *Xho*I for MreBR. The Glutathion S-Transferase (GST) gene for fusion protein construction was amplified by PCR using the primers GSTF and GSTR. Each primer contains a restriction site for *Nde*I. The vector pETgst to express His-GST as a control for pull-down assay was also constructed, where GST gene was amplified using the primer GSTF and the primer GSTRX. All the primers were designed in the DNA Star Lasergene® software. The *mreB* gene was amplified from *H. pylori* 26695 genomic DNA while the GST tag was amplified from the *pAcGHLTB* vector.

The *mreB* and *gst* genes were firstly cloned into pSCA Strataclone® cloning vector and transformed into *E. coli DH5α*. Then *mreB* and *gst* genes were sequentially sublconed into the modified *pET19b* expression vector which contains the 10 × His Tag by the use of restriction sites, *Xho*I and *Nde*I. Two final constructs (*pETmreBr* and *pETgst*) were obtained, respectively containing the genes of recombinant proteins 10 × His-GST-MreB and 10 × His-GST (Fig. S1). We used two tags (poly-Histidine and GST) in tandem for the fusion proteins that provided facility for purification and pull-down assay. The kit BigDye® and Applied Biosystem® sequencer of 4 capillaries model 3130 were used for sequencing to confirm all the constructs.

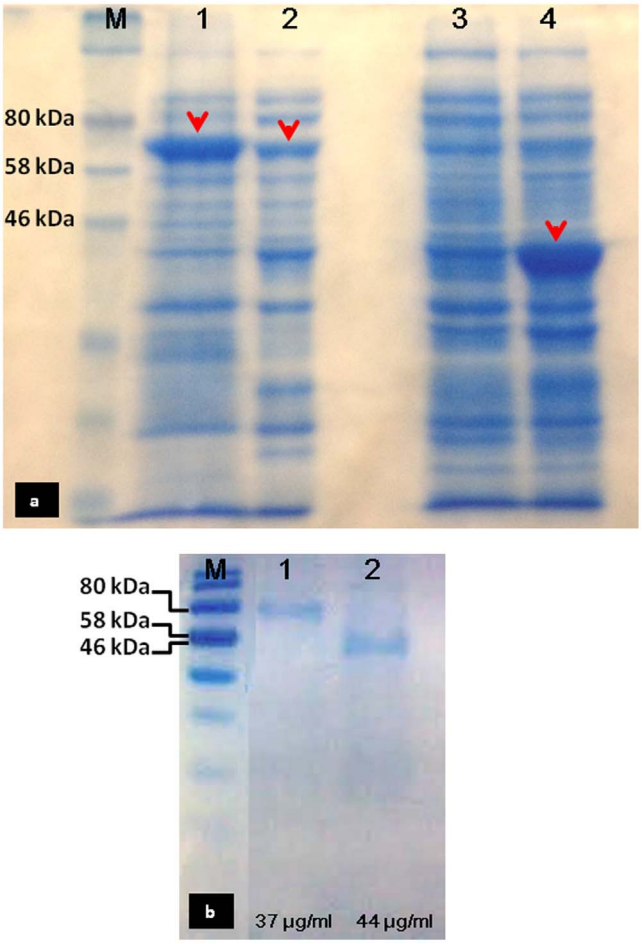

Fig. 1. The overexpression and purification of recombinant proteins (MreB, GST) as shown by polyacrylamide gel electrophoresis. a) In lanes 1 and 2 the red arrow indicates the overexpression of the recombinant MreB of *H. pylori* 26695 (77 KDa) in both soluble and insoluble fractions, respectively. Lane 4 shows the overexpression of recombinant GST (40KDa) in soluble fraction (indicated with an arrow) while line 3 shows that recombinant GST was not overexpressed in the insoluble fraction. The letter "M" represents protein marker (P7709S, Biolabs). b) Lane 1 shows the recombinant MreB purified from soluble fraction while lane 3 shows the recombinant GST purified from soluble fraction. The concentration of each recombinant protein purified is indicated at the bottom of gel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Protein overexpression and purification

The plasmids *pETmreBr* and *pETgst* were respectively transformed into *E. coli Codon Plus* strain. The recombinant MreB (10 × His-GST-MreB) overexpression was induced for 4 h by adding 1 mM IPTG when the OD_{600} reached 0.6 at 37 °C while recombinant GST (10 × His-GST) was induced for 2 h by adding 1 mM IPTG when the OD_{600} reached 1.2. The cells were collected by brief centrifugation at 14,000 rpm and lysed by sonication. Proteins were purified using His Trap HP columns according to the manufacturer's instruction (General Electric®). The results were visualized with 10% acrylamide gels for SDS-PAGE. The fusion proteins were purified from the soluble fraction. The recombinant MreB from soluble fraction was concentrated in an Amicon® Ultra 4 ml Centrifugal Filter column for reducing the solvent 4 times. Then, both fusion proteins were further dialyzed in a phosphate buffer 50 mM (KH_2PO_4 25 mM and K_2HPO_4 25 mM) to reduce the imidazole to a final molarity of imidazole < 0.01 mM. A Spectra/Por® cellulose membrane with 29 mm of diameter and a pore of 0.01 μm was used for dialysis.

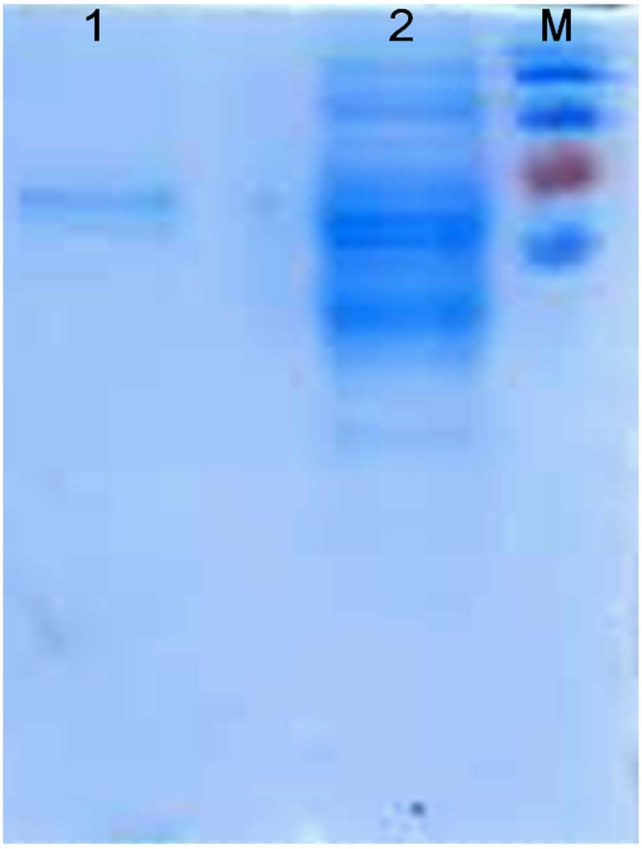

Fig. 2. The pull-down result with recombinant MreB from *H. pylori* 26695 and its control shown by polyacrylamide gel electrophoresis. The control using the recombinant GST is shown in lane 1. Lane 2 shows the bands of pull-down proteins in *H. pylori* 26695 test. The letter "M" represents protein marker (PageRuler™ Prestained Protein Ladder).

2.4. Pull-down assay and protein identification by mass spectrometry

The pull-down assay was carried out according to previously reported protocol (Ranjan et al., 2013). *H. pylori* 26695 cells were lysed in lysis buffer (100 mM NaH_2PO_4, 100 mM NaCl, 10 mM imidazole, 1% Tween 20 and 10 μg/ml PMSF). Then, 50 μl of Genescript® microspheres coated with nickel was placed in a microtube, by adding 10 mg of recombinant GST and the *H. pylori* 26695 cell lysate (1×10^{10} cells). After mixed incubation for 2 h on ice, the proteins that could interact with recombinant GST binding to the microspheres were eliminated by centrifugation (14,000 rmp). This step is called pre-cleaning. The supernatant was placed into two microtubes. It was added to each tube with another 50 μl of microspheres and 10 μg of recombinant MreB or recombinant GST as a control purified from the soluble fraction. After mixture and 2 h of incubation on ice, the recombinant proteins and its complexes attached to the microspheres, which were separated by centrifugation (14,000 rmp, 1 min). The microspheres were washed with wash buffer (100 mM NaH_2PO_4, 100 mM NaCl and 20 mM imidazole) and then were eluted with elution buffer (100 mM NaH_2PO_4, 100 mM NaCl and 500 mM imidazole). The eluted proteins were observed on 10% acrylamide gel. The protein bands on the gel were cut off and purified, and then the proteins were identified by MALDI-TOF-MS.

2.5. Protein-protein interaction experiment in E. coli

For confirming the interactions between MreB and other proteins obtained in the pull-down assay from lysate of *H. pylori* 26695, we used the Bacterial Two-Hybrid system (BACTH system) kit (Euromedex, France) for the assay. As MreB was found to interact with MreC in other bacteria (Kruse et al., 2005; Kawai et al., 2009), we also added an assay to test this interaction in *H. pylori*. The 25 proteins from the pull-down products were randomly chose for evaluation except nine pathogenesis process-associated factors. The genes *mreB*, *mreC* and 25 other genes were cloned into the cloning vector *pSCA* (Strataclone®). Then, the gene *mreB* was subcloned into *pKT25* vector (*pKT25-mreB*) for fusing MreB to the adenilate cyclase subunit T25 of *Bordetella pertussis*. The primers MF2H, MR2H containing the restriction sites, *Pst*I and *Eco*RI, respectively, were designed for the MreB amplification and the fusion protein construction. Each of 25 genes or *mreC* was then subcloned into pUT18C vector for fusing to adenylate cyclase subunit T18. All the primers for other gene amplification were supplied in Table S1.

E. coli BTH101 was co-transformed with plasmids *pKT25-mreB* and *pUT18C* that contained the insert of gene tested. The negative control of BACTH system was used for co-transformation with the vectors pKT25 without insert and *pUT18C* that contains the gene, not *pKT25-mreB* and *pUT18C*. The transformants were selected on LB agar plates supplemented with 40 μg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal), 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 100 μg/ml ampicillin and 50 μg/ml kanamycin. The transformants in deep-blue color after 24 h indicate the interaction is very possibly positive. The β-galactosidase activity in liquid LB was then measured for quantification of the functional complementation mediated by interaction between two proteins. Galactosidase activity is defined as units/mg of dry weight bacteria and 1 unit corresponds to 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute at 28 °C. The galactosidase activity 5-fold higher than negative control was considered as a positive interaction according to the manual. Co-transformation into *E. coli* BTH101with pKT25zip and pUT18Czip (which contain a leucine zipper that ensure the complementation of T25 and T18 subdomains) served as positive control in the test. The two hybrid assay and β-galactosidase activity determination were carried out at least three times.

3. Results and discussion

3.1. Construction of expression vectors, overexpression and purification of recombinant proteins

Two plasmids for expression of recombinant proteins His-GST-MreB and His-GST were respectively constructed as shown in Fig. S1. Both recombinant proteins were overexpressed in *E. coli Codon Plus* strain. Recombinant MreB expressed much less amount in soluble fraction than in the insoluble fraction whereas the recombinant GST was expressed very soluble (Fig. 1. Panel a). Both recombinant MreB and GST were purified from soluble fraction. The quantity of recombinant MreB purified from soluble fraction was so small that repeated enrichment was required to obtain (Fig. 1. Panel b).

3.2. The pull-down assay and the identification of proteins associated with MreB complexes in H. pylori 26695

Both recombinant MreB and GST purified from soluble fractions were used to perform the pull-down assay. After pre-cleaning, the pulled-down products and their control were shown in Fig. 2. The protein bands on acrylamide gel from pull-down products for recombinant MreB were separately cut out and purified for identification by mass spectrometry. The 85 proteins were determined (Table S2). Nine proteins (VacA, GroEL, UreB, HylB, HydB, KatA, ThrC, TsaA and SpeA) that are associated in the pathogenesis processes were found in the products. Comparing our results with the proteins produced by pull-down experiments or TAP from *E. coli* and *B. subtilis* (Butland et al., 2005; Arifuzzaman et al., 2006; Kawai et al., 2011), a few proteins were shared but a big difference was also shown, indicating that the MreB interactome in one species is very different from another (Fig. S2).

Meanwhile, our pulled-down products did not contain any impor-

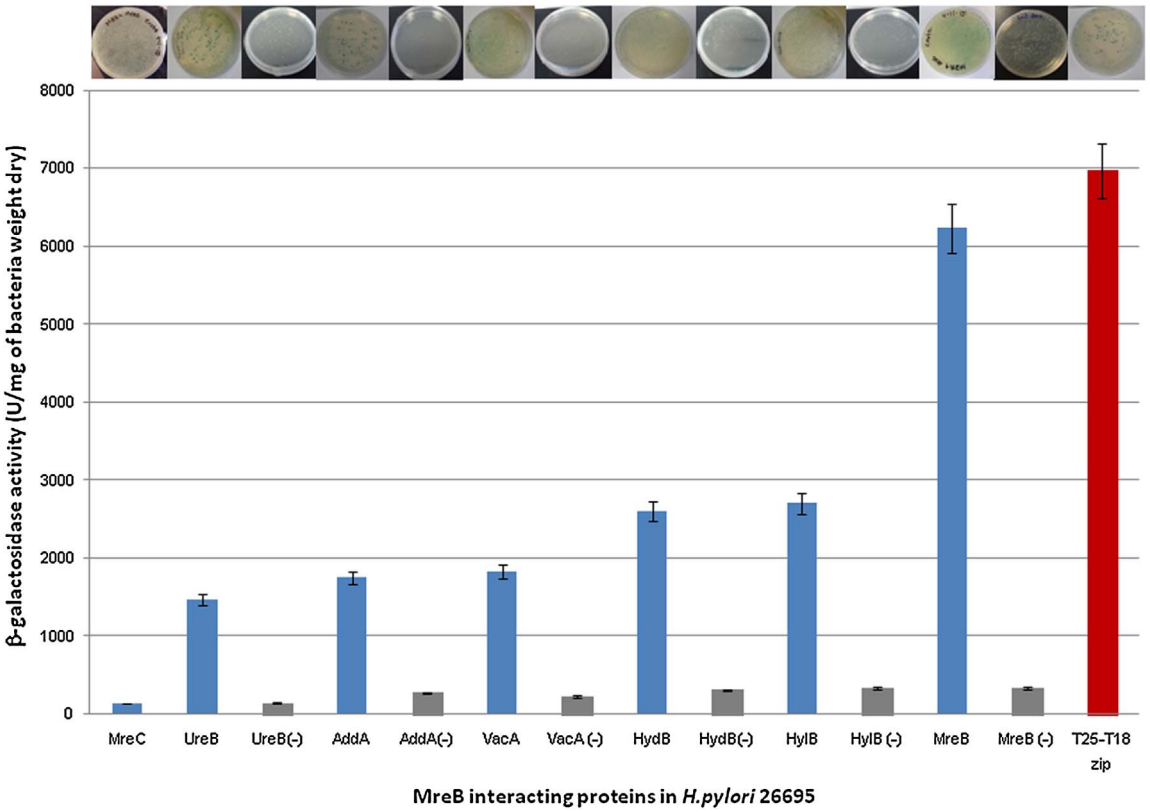

Fig. 3. Protein interactions with MreB determined by BACTH system. At the top of graph, the trial results in petri dishes are shown. The dish with blue colonies indicate a positive interaction while the dish with white colonies mean a negative interaction. The solid bars indicate β-galactosidase activity determined by BACTH system for the positive protein interactions. Each bar represents the average of three trials. Error bars show the standard deviation from the average. The blue shows MreB positive interactions, the gray shows the negative controls (−) and the red indicates the positive control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tant proteins involved in the morphogenesis process, unlike the cases of TAP or pull-down tests in either *E. coli* or *B. subtilis* (Butland et al., 2005; Kawai et al., 2011). For example, the proteins, MurG (UDP-*N*-acetylglucosamine-*N*-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase) and MurF (UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase), involved in peptidoglycan biosynthesis and determinant for cell shape (Favini-Stabile et al., 2013), are present in *E. coli* or *B. subtilis* assays but absent in *H. pylori* assay. It is noteworthy that RodZ, a main protein involved in cell morphogenesis (Bendezu et al., 2009; van den Ent et al., 2010), is not encoded by *H. pylori* genome and thus absent in the assay. The proteins, AmiA, Ccrps, HdpA, Csd1-5, CcmA were confirmed to affect the cell shape in *H. pylori* (Chaput et al., 2006; Waidner et al., 2009; Bonis et al., 2010; Sycuro et al., 2010; Sycuro et al., 2012), but none of them were in our pull-down products.

3.3. MreB interaction with 25 proteins tested by BACTH system

Taking into consideration that the pulled-down products were strongly affected by the salt concentration of wash buffers so that non-specific or sticky proteins could occur in 85 proteins of our experiment due to the presence of trace bands in the control, it is necessary to further confirm the direct protein interactions by BACTH system. In order to get convincing results for protein interaction, in addition to the positive and negative controls required for the BACTH system, we added two interaction tests related to MreB for *H. pylori*. One interaction is MreB-MreB due to the formation of MreB filament in *H. pylori* and the other interaction is MreB-MreC as a usual interaction in other bacteria, although MreC was not present in the our pulled-down products. The interaction MreB-MreB was confirmed, indicating that this system works well to evaluate for *H. pylori* proteins. Then, the tests for MreB interaction with MreC and each of 25 proteins were performed according to BACTH system procedure. The results showed that MreB did not interact with MreC but directly interacted with each of the five proteins (UreB, VacA, HydB, HylB and AddA) (Fig. 3), although they showed less galactosidase activity than MreB-MreB.

It was reported that MreB mutant did not apparently affect the cell shape in *H. pylori* (Waidner et al., 2009). MreB and MreC are located at the same operon in most of bacteria including *H. pylori*. The direct interaction between them was confirmed in *E. coli* (Kruse et al., 2005), but MreC was not present in the pulled-down products in *H. pylori*, concordant with the result from our two-hybrid assay. In *B. subtilis* and *H. pylori*, MreC is connected to the Penicillin-Binding Proteins (PBPs) that participate in cell morphogenesis (El Ghachi et al., 2011; Kawai et al., 2011). Taking together the information above, our results indicated *H. pylori* MreB could not be involved in cell morphogenesis (Waidner et al., 2009) possibly due to the absence of interaction with MreC.

Three (UreB, VacA, HydB) of the nine proteins involved in pathogenesis process presented direct interaction with MreB. VacA is a main toxin of *H. pylori* to human cells and MreB showed antigenic properties in serum samples from patients with gastric cancer or infection (Gao et al., 2009; Formichella et al., 2013), indicating that MreB could participate in VacA pathogenesis process, *e.g.*, VacA transportation. UreB is one structural subunit of urease (Marais et al., 1999; Schoep et al., 2010; Yamaoka, 2010). The TAP products of UreB from *H. pylori* contained MreB (Stingl et al., 2008), implying that MreB exists in the complex of UreB, exactly in concordance with our pull-down experiment. The direct interaction MreB-UreB gives a possible reason why urease activity was affected by *mreB* mutant (Waidner et al., 2009).

Furthermore, the interactions of MreB with VacA and UreB analyzed

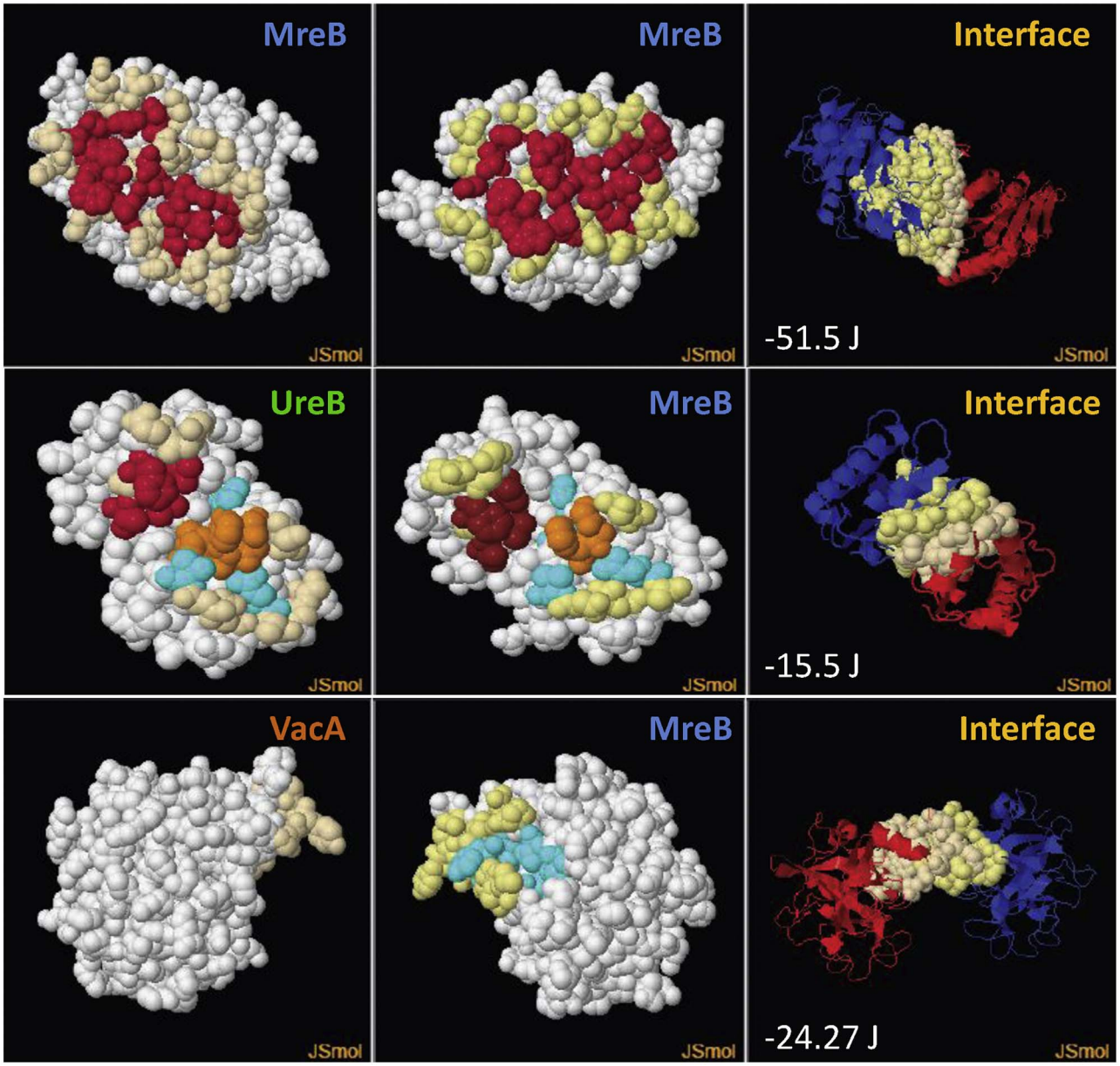

Fig. 4. Interfaces of MreB interactions with VacA, UreB and itself predicted *in silico*. In the 3D structure of MreB, UreB and VacA, the hot spots of interaction with MreB are indicated. In addition, the interface and energy of each interaction is shown.

in silico also showed the possible interactions of MreB with VacA and UreB. A MreB homology model of *H. pylori* was generated using Geno3D software (Fig. S3) based on crystallographic structure of both *Thermotoga maritima* (PDB code 1JCF) and *Caulobacter crescentus* (PDB code 4CZE). The crystallographic structures of virulence factors in *H. pylori*, urease (PDB code 1E9Y) and VacA (PDB code 2QV3), were previously reported (Ha et al., 2001; Gangwer et al., 2007). The software Prism 2.0 (Ogmen et al., 2005) was used for determining if the tridimensional structure of MreB could interact *in silico* with itself as well as with other two virulence factors. The homology modeling and their interactions were observed with the help of PyMOL software (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC). The free energy for interaction showed −24.27 kcal/mol for MreB-VacA, −15.1 kcal/mol for MreB-UreB and −51.5 kcal/mol for MreB-MreB, implying the possible interactions with MreB (Fig. 4). The putative residues of interaction between these proteins are shown in Fig. S4, implying that these two interactions are involved in different motifs of MreB. This result is consistent with that of our BACTH tests and indicates that the MreB could interact with both UreB and VacA *in vivo*.

The hydrogenase subunit β (HydB), working together with other proteins such as HydA and HydC, has a strong impact on redox homeostasis for bacteria that live in the stomach and is a major determinant of inflammation (Pich and Merrell, 2013; Wang et al., 2016). It has been further observed that *H. pylori* wild-type strain 7.13 induced gastric cancer in 50% of gerbils but its Δ*hyd* strain did not (Wang et al., 2016), indicating the important function of hydrogenase in gastric cancer development. Thus, the interactions MreB-HydB could affect HydB function in *H. pylori* infection. Taken all results together, it demonstrates that MreB could have a role in the pathogenesis of *H. pylori*.

MreB is involved in chromosome segregation in *H. pylori* and other bacterial species such as *E. coli, B. subtilis* and *C. crescentus* (Formstone and Errington, 2005; Gitai et al., 2005; Madabhushi and Marians, 2009; Waidner et al., 2009). The process of chromosomic segregation is related to DNA repair and recombination (Carrasco et al., 2004). AddA (HP1553), previously called RecB, AddB (HP1089) and HP0275 (AddB previously annotated), were found in the pulled-down products of *H.*

pylori while RecA (recombinase subunit α) occurred in the test for *E. coli*. AddAB in *H. pylori* is functionally similar to RecBCD in *E. coli* (Amundsen et al., 2008) and are required for DNA repair, recombination, and mouse infectivity (Amundsen et al., 2009). The positive MreB-AddA interaction implies the possibility that MreB could participate in this cell process via the functional complex MreB-AddAB-RecA in *H. pylori*.

Meanwhile, we compared our results with those from previous protein-protein interaction studies in large scale for *H. pylori* (Rain et al., 2001; Pyndiah et al., 2007; Häuser et al., 2014). The hypothetical proteins (HP0339 and HP0947), chromosome partitioning protein ParB and urease subunit UreE presented interactions with MreB, however, these proteins did not occur in our pull-down test. These interaction experiments were performed in yeast system, not in *E. coli*, but this observation is still a surprise to us. In our experiments, we used not only the control for BACTH system, but also the reactions MreB-MreB, MreB-MreC of *H. pylori*, indicating the reaction system works properly. It could be some other factors such as distinct chaperones required for the interactions in these two model cells (*E. coli* and yeast) resulting in determination of distinct protein-protein interactions. Furthermore, it is somehow unclear if His-GST tag or pH environment affects the binding ability of functional proteins in the MreB complexes. Hence, the novel methods for detecting new interactions could be needed.

In summary, bacterial MreB has functional diversity so that the distinct interactomes of this protein were shown in *E. coli*, *B. subtilis* and *C. crescentus*. Here, the pulled-down products of MreB from *H. pylori* provided protein candidates for direct interaction evaluation. New interaction proteins with MreB were demonstrated by BACTH system. All the results indicated that MreB in *H. pylori* may not be involved in cell morphogenesis process, at least not via the pathway of MreC as in many other bacteria, but may participate in pathogenesis. Further studies are needed to elucidate the molecular mechanisms of pathogenesis by MreB.

Acknowledgments

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

This article does not contain any studies with human participants or animals performed by any of the authors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2017.04.008.

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