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Selection of LigA-Binding Peptides by Bacterial Display

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Abstract

Leptospirosis is a severe disease of human and animals caused by pathogenic *Leptospira* spp. Previous findings reveal that the organisms express leptospiral immunoglobulin-like protein A (LigA) on their surfaces only *in vivo*, which is believed to serve as a virulence factor for host cell attachment and invasion. Recently, the LigA protein has become a potential target for laboratory investigation and vaccine development. In the present study, screening and selection of LigA-binding peptides have successfully been carried out with the aid of bacterial display system. After five rounds of bio-panning, peptide sequences of MSQRRAIPTGSI, ILSSPKLRADDL and VVKAGMMPVASL were found to exhibit high affinity to bind to immobilized LigA. No consensus sequence was observed among 12 isolated bacterial clones. The peptide MSQRRAIPTGSI demonstrated the highest binding activity to LigA with no cross-reactivity to the bacterial lipopolysaccharide. Emphasis can be taken that the LigA-binding peptides showed sequence similarity to those of the binder molecules such as ABC transporters, Leucine-rich repeats (LRR), and Zinc-finger protein. Interestingly, both the ABC transporters and the Leucine-rich repeats engage with several biological processes at the cell membranes. Moreover, it is well-established that the LRR plays imperative roles in protein-protein interaction, particularly between bacterial peptide and host receptors. Therefore, our findings lend support to the evidences that expression of the LigA on bacterial surfaces possessed binding capability to host proteins, receptors or extracellular matrices and mediated their attachment on the host cell membranes. Taken together, significant from this work not only gains insights into the plausible binding sites of LigA on the host cells but

also opens up a high feasibility to utilize the LigA-binding peptides as diagnostic and therapeutic tools in the future.

Keywords: LigA, *Leptospira* spp., Bacterial display, Slide-binding assay

1. Introduction

Leptospirosis is a re-emerging severe disease caused by spirochetes of the pathogenic *Leptospira* spp. (for recent review please see Cruz et al., 2009). The virulence strain of this organism can cause multi-organs failure in both human and animals owing to the capability to penetrate and replicate in many tissues, e.g., kidney, liver, spleen, lung, eyes and central nervous system. Such serious complications can be overcome by early and accurate detection followed by proper treatment. Therefore, attempts have been geared towards the development of DNA amplification methods (Fernandes et al., 2008; Lin et al., 2009; Stoddard et al., 2009), immunological assays (Chirathaworn et al., 2007; Dey et al., 2008; Lin et al., 2008a; Senthilkumar et al., 2008; Srimanote et al., 2008) and microscopic testing (Chandrasekaran and Gomathi, 2004) to pave the way of achieving efficient diagnostic strategy.

Among the antigens used for laboratory investigations, leptospiral immunoglobulin-like protein A (LigA) has received an increased attention due to the occurrence only in pathogenic leptospires (Palaniappan et al., 2004). It has been identified to encompass tandem repeats of bacterial immunoglobulin-like (Big) domain (Palaniappan et al., 2002). Expression of the LigA as a surface-exposed protein can be detected only *in vivo* condition, which plays important roles in host cell attachment and invasion (Choy et al., 2007; Matsunaga et al., 2003). More importantly, diminution of LigA expression is correlated with the loss of ability to produce lethality in host (Matsunaga et al., 2003). Therefore, the use of LigA as potential target for laboratory diagnosis of leptospirosis and as vaccine candidate have been reported (Cerqueira et al., 2009; Faisal et al., 2008; Koizumi and Watanabe, 2004; McBride et al., 2009; Palaniappan et al., 2004; Palaniappan et al., 2006; Srimanote et al., 2008).

Bacterial surface display has become an invaluable tool for selection of peptides that bind specifically to the target molecules. Display of short peptide library on the surfaces of Gram positive and Gram negative bacteria has successfully been carried out by many systems, e.g., using protein A, outer membrane protein, lipoproteins, auto-transporters, and subunits of surface appendages (Kronqvist et al., 2008; Samuelson et al., 2002). Applications can be established in many biotechnological and medical aspects such as bacterial targeting, epitope mapping, protein engineering, biocatalysis, bioremediation, cellular adsorbents and vaccine development (Daugherty, 2007; Samuelson et al., 2002).

In the present study, we utilize the bacterial display technology to search for peptides possessing high affinity to the LigA protein. A random dodecapeptide library has been used to select bacterial clones with LigA-binding specificity. Cross-reactivity of the bacterial clones with lipopolysaccharide has further been analyzed. Feasibility of using such peptides as potential tools for diagnostic and therapeutic purposes has been discussed.

2. Materials and Methods

2.1. Materials

Bovine serum albumin (BSA), casein and Tween 20 were purchased from Sigma. Purified LigA was a generous gift from Assistant Professor Dr. Potjanee Srimanote (Srimanote et al., 2008). All other chemicals and reagents were of analytical grade and commercially available.

2.2. Media Preparation

Stock M9 salts (pH 7.4, 10 X concentrations) were prepared by dissolving 60 g of Na_2HPO_4, 30 g of KH_2PO_4, 5 g of NaCl, and 10 g of NH_4Cl in 1 L of deionized water. IMC medium is M9 supplemented with 0.2% casamino acids, 0.5% glucose, and 1 mM $MgCl_2$. RM medium is M9 supplemented with 2% casamino acids, 1% glycerol, and 1 mM $MgCl_2$. RMG-Amp plates is M9 medium that contained 2% casamino acids, 0.5% glucose, 1 mM $MgCl_2$, 100 µg/ml ampicillin and 1.5% agar.

2.3. Bio-Panning

A peptide display library, FliTrx™ (Invitrogen, Carlsbad, CA), was used to identify peptides providing high binding specificity to LigA in the bio-panning process. The FliTrx is a bacterial (*E. coli*) display system that expresses random dodecapeptides on the flagella tip. To the procedure herein, 20 µg/ml purified LigA protein was coated onto 60 mm tissue culture plate for 1 hour and subsequently incubated with blocking solution (IMC medium containing 100 µg/ml ampicillin, 1% nonfat dry, 150 mM NaCl and 1% α-methyl mannoside). One vial of the FliTrx peptide library was taken from the freezer and thawed at room temperature. The FliTrx peptide library was inoculated in 50 ml IMC medium containing 100 µg/ml ampicillin (no tryptophan) and the cells was then grown with shaking (225-250 rpm) to saturation (OD600 ~3) for overnight at 25°C. Expression of the FliTrx library was induced by adding 1×10^{10} cells of the overnight culture to 50 ml IMC medium containing 100 µg/ml ampicillin and 100 µg/ml tryptophan. The culture was grown at 25°C with shaking for 6 hours. Next, 10 ml was removed from the induced culture, added into the centrifuge tube containing 0.1 g dry milk, 300 µl 5 M NaCl, 500 µl 20% α-methyl mannoside, and swirled thoroughly. The α-methyl mannoside is required to prevent selection of fimbriated *E. coli* since they can usually adhere to antibodies via lectin-like interactions. Therefore, supplementation of α-methyl mannoside to the cultured library and in the washing and blocking solutions prevents the isolation of false positive clones. The blocking solution was removed from the LigA plate (as described earlier) and subsequently added the 10 ml induced cell solution containing 0.1 g dry milk, 300 µl 5 M NaCl, 500 µl 20% α-methyl mannoside. The plate was gently agitated at 50 rpm for 1 minute and then incubated at room temperature for 1 hour. It is very important to handle the plates gently in order to prevent shearing of flagella, which results in the loss of positive clones. The plate was further washed gently with 10 ml washing solution (IMC medium containing 100 µg/ml ampicillin, 1% α-methyl mannoside) at 50 rpm for only 5 minutes to remove the unbound bacterial cells. The washing step was repeated for 4 more times. After the fifth wash was decanted, the cells were eluted by vortexing the plate for 30 seconds into the small volume of residual washing solution. The plate was added with 10 ml of IMC medium containing 100 µg/ml ampicillin (no tryptophan) and the solution was pipetted to a 50 or 125 ml culture flask. Ten milliliters of the solution was cultured with shaking (225-250 rpm) at 25°C for 15 hours, returned to induction of library, and repeated all steps to this point for four more times. After the fifth panning, the cells were grown for overnight. Then, the cells was spread onto RMG plates containing 100 µg/ml ampicillin and incubated for overnight at 30°C for further selection of isolated colony.

2.4. DNA Sequence Analysis

Selected colonies were inoculated in 5 ml of RM medium containing 100 µg/ml ampicillin. It is very important to use RM medium for this step to obtain high yields of plasmid DNA. The cultures were grown for 16-24 hours at 30°C with shaking. Plasmid DNA was recovered using the NucleoSpin Plasmid Extraction Kit (Macherey-Nagel, Germany) and the nucleotide sequences of the inserts were assayed using the forward (5´-ATTCACCTGACTGACGAC-3´) and reverse (5´-CCCTGATATTCG-TCAGCG-3´) primers.

2.5. Slide-Binding Assay

To identify the bacterial clones those possessed LigA-binding activity, slide binding assay was developed. Experimentation was carried out by coating LigA on glass slide for 1 hour and 30 minutes and then the slide was subsequently incubated with blocking solution (1% BSA, 0.05% Tween 20) for 45 minutes. After that, induced cells (5×10^8 cfu/ml) were applied onto the slide for 1 hour and 30 minutes at room temperature. Unbound bacteria were removed using washing solution containing 0.05% Tween 20. Gram staining was performed and the bacterial cells those specifically bound onto the slide were counted.

2.6. Computational Analysis of the Protein Properties

ProtParam (http://br.expasy.org/tools/protparam.htm) was used as a tool for the calculation of short peptide properties including the molecular weight, pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY).

2.7. Cross-Reaction Analysis with Lipopolysaccharide

To further investigate whether the LigA-binding peptide exhibited cross-reactivity to other biomacromolecules, the bacterial clone that possessed the highest binding activity to LigA was incubated with purified lipopolysaccharide of *Salmonella enteritidis* in the indirect competitive slide-binding assays. Briefly, 10 µg/ml of purified LigA was coated on glass slides. Then, the slide was added with blocking solution and further incubated for 45 minutes. Induced bacterial cells (5×10^8 cfu/ml) was mixed thoroughly with 10 µg/ml purified lipopolysaccharide of *S. enteritidis* and gently agitated at 50 rpm for 1 hour and 30 minutes at room temperature. The solution was further applied onto the slide. After binding process, the slide was stained as mentioned above.

3. Results and Discussion

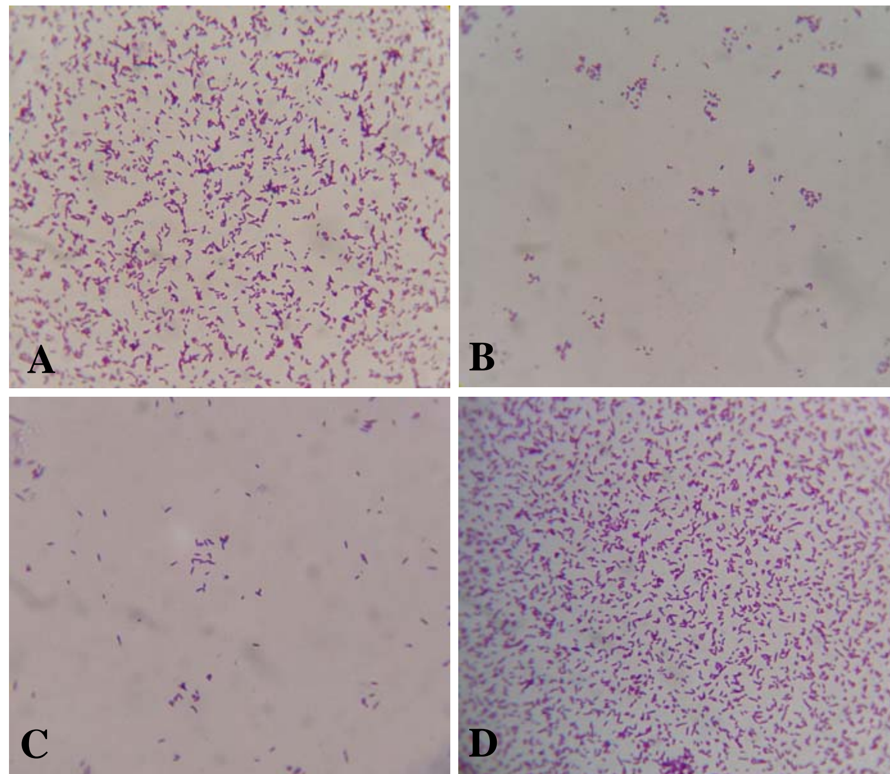
3.1. Screening of LigA-Binding Peptides by using Bacterial Display

The FliTrx library of the random 12-amino acid peptide library was subjected to bio-panning process on immobilized purified LigA. After five rounds of biopanning, 40 bacterial display clones were randomly selected on the RMG plates. Slide-binding assays were performed in order to identify the bacterial clones those possessed specific binding capability to LigA. As represented in Figure 1D, numerous Gram negative bacilli bound specifically to the immobilized LigA. The same degree of binding was observed in the presence of induced cells and unblocked LigA (Figure 1A). This indicates that the slide was fully occupied by the immobilized LigA. As negative control, a very few amount of induced cells was found to adsorb non-specifically on the bare slide (Figure 1C). In addition, un-induced bacterial cells possessed low binding capability to the immobilized LigA (Figure 1B). Among 40 clones, only 12 exhibited significant LigA binding activity (Figure 2). These clones were subjected to the DNA sequencing process and the DNA sequences were decoded to yield amino acid sequences as shown in Table 1. It was found that the bacterial colonies No. 14, 9 and 29 (with the sequences of MSQRRAIPTGSI, ILSSPKLRADDL and VVKAGMMPVASL, respectively) provide the highest binding activity to immobilized LigA. No consensus sequence and differences in protein property were observed among these 12 clones. This is not surprising since the property of LigA resembled those of the lipopolysaccharides (LPS) or endotoxin in which binding of the peptide display systems to the LPS was observed to be non sequence-specific and showed sequence diversity in many studies (Noda et al., 2001; Thattiyaphong et al., 2006; Thomas et al., 2003; Zhu et al., 2003). In certain circumstances, presentation of the whole three-dimensional conformations of LPS on the epoxy bead may improve the binding selectivity (Kim et al., 2005).

3.2. Cross-Reaction with Lipopolysaccharide

Clone No. 14 (MSQRRAIPTGSI) that provided the highest binding capability to LigA was subjected to the indirect competitive slide-binding assays with lipopolysaccharide. It was found that no significant different in the binding capability to immobilized LigA was found in the presence and absence of lipopolysaccharide (Figure 3). Our findings confirmed the high specificity of the peptide MSQRRAIPTGSI to bind to LigA with no cross-reactivity against lipopolysaccharide.

Figure 1: Screening of bacterial display clones possessing specific binding capability to LigA by slide binding assays.



Note : A: Induced bacterial cells + unblocked immobilized LigA, B: un-induced bacterial cells + blocked immobilized LigA, C: induced bacterial cells + un-immobilized LigA, D: induced bacterial cells + blocked immobilized LigA.

Figure 2: Binding activity of bacterial clones to immobilized LigA.

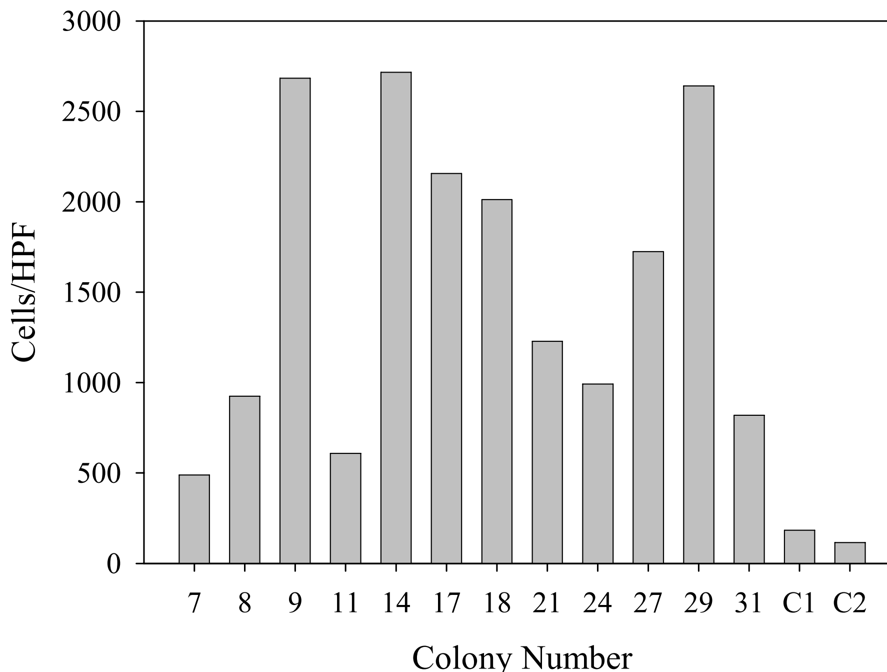
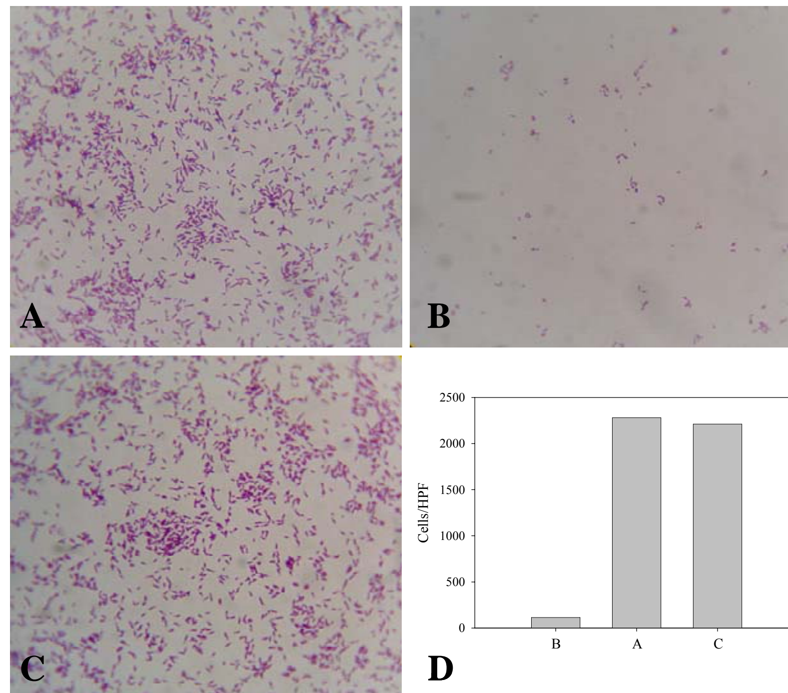


Table 1: Peptide sequences and properties of LigA-binding peptides.

Sample No.	Sequence	Cell Count (Cells/HPF) Control = 183	M.W.	pI	Aliphatic Index	Hydro-Pathicity	Neg. (-) Charge	Pos. (+) Charge
14	MSQRRAIPTGSI	2716	1316.5	12.00	105.83	-0.125	1	2
9	ILSSPKLRADDL	2684	1327.5	5.96	138.33	-0.075	2	2
29	VVKAGMMPVASL	2641	1202.5	8.72	121.67	1.425	0	2
17	TDAQVRLDAMVS	2156	1305.4	4.21	97.50	0.100	2	1
18	LNRDVWRGSAEL	2012	1415.5	6.07	97.50	-0.667	2	2
27	ALHLGRGAVDRG	1724	1221.3	9.65	105.83	-0.125	1	2
21	EDGGEIRAARRG	1228	1286.3	6.28	49.17	-1.425	3	3
24	VIAGCNQRARSI	992	1287.5	10.35	105.83	0.175	0	2
8	NRVEGDSAIIGE	925	1259.3	4.43	97.50	-0.425	3	1
31	PGRRESLVLGFS	819	1317.5	10.03	89.17	-0.158	1	2
11	FSHRPGKRGKDG	608	1341.5	11.00	0.00	-2.025	1	4
7	EGVAAGGVLTRG	489	1086.2	6.10	97.50	0.458	1	1

Figure 3: Cross-reaction testing with the lipopolysaccharide.



Note: A: Induced bacterial clone No.14 + 10 μg/ml immobilized LigA, B: Un-induced bacterial clone No.14 + 10 μg/ml immobilized LigA, C: Induced bacterial clone No.14 pre-incubated with 10 μg/ml lipopolysaccharide + 10 μg/ml immobilized LigA, D: Comparison of cell counts in each condition.

3.3. Sequence Similarity of LigA-Binding Peptides with Protein Database

Sequence similarity of the LigA-binding peptides (MSQRRAIPTGSI, ILSSPKLRADDL and VVKAGMMPVASL) was further analyzed by comparing with the protein database using BLASTP 2.2.16 (http://www.ncbi.nlm.nih.gov/BLAST). Results revealed that most of the peptides show high sequence similarity (~65-85%) to those of the binder molecules such as ABC transporters, Leucine-rich repeats (LRR), and Zinc finger proteins. It is interesting to mention that both of the ABC transporters and the Leucine-rich repeats involved with biological processes at the cell membranes. The ABC transporters (ATP-binding cassette transporters) are transmembrane proteins that facilitate translocation of various substrates (e.g. metabolic products, lipid, ions and drugs) across membranes of prokaryotes and eukaryotes by utilizing the energy of ATP hydrolysis (Rees et al., 2009). The LRR (Bella et al., 2008) (found in tyrosine kinase receptor, cell adhesion molecules, extracellular matrix-binding glycoproteins etc.) plays imperative roles in protein-protein interaction in many systems. For instances, it serves as common pattern recognition motifs for binding interaction between bacterial peptide and host receptors (Loimaranta et al., 2009). It has also been shown to be participated in the host defense mechanisms of plants (resistance genes) and mammals (Toll-like receptors and nucleotide-binding oligomerisation domain proteins), in which triggering of specific pathogen-associated molecules and activation of innate immune responses have been accounted (Kedzierski et al., 2004). The Zinc finger proteins are small protein domains capable of coordinating one or more zinc ions to stabilize their structures. Such proteins are known to function as transcriptional regulations and serve as interaction modules that bind nucleic acids, proteins or small molecules (Dent et al., 2007; Song et al., 2003; Tsai and Reed, 1998). Roles of the Zinc finger proteins on the correlation between LigA and host proteins remain questionable. However, it is conceivable that the role of mediating protein-protein interactions may have taken place (Dai and Liew, 2001; Liu et al., 2008). Taken altogether, our findings lend support to the previous conclusion that expression of the LigA on

Leptospira' cell surfaces possessed capability to bind to peptides, receptors or extracellular matrices and mediated their attachment on host cell membranes (Choy et al., 2007; Lin et al., 2008b; Matsunaga et al., 2003). Moreover, a better understanding on the sequence similarity of LigA-binding peptides not only gains insights into the plausible receptor sites and mode of binding interaction between LigA and host cells, but also expands further applications as a potential inhibitor for therapeutic purposes.

4. Summary and Future Perspectives

Herein, selection of dodecapeptides those possessed binding avidity to LigA was successfully carried out using a bacterial display system. After the fifth round of bio-panning, the LigA-binding peptide (MSQRRAIPTGSI) exhibited very high affinity to the immobilized LigA with no cross-reactivity to the bacterial lipopolysaccharide. Such findings open up a high feasibility to develop the LigA-binding peptides as a diagnostic tool for leptospirosis. This can be realized either by competitive binding assay using synthetic peptides or fusing the LigA-binding peptides with reporter molecules (e.g. fluorescent proteins or enzymes) for antibody or antigen detection, respectively. Moreover, further investigations particularly on the inhibitory effect of synthetic peptides on the binding interaction between LigA and host cells are needed to be performed.

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