PHAGE DISPLAYED ANTIBODY AGAINST *BRADYRHIZOBIUM YUANMINGENSE* STRAIN DOA9 AND ITS POTENTIAL APPLICATION IN AGRICULTURE

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แอนติบอดี้แสดงบนผิวเฟจที่จำเพาะกับ *BRADYRHIZOBIUM YUANMINGENSE* สายพันธุ์ DOA9 และศักยภาพในการประยุกต์ใช้ในเชิงการเกษตร

นายวู เหงียน ซวน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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วู เหงียน ซวน : แอนติบอดี้แสดงบนผิวเฟจที่จำเพาะกับ *BRADYRHIZOBIUM YUANMINGENSE* สายพันธุ์ DOA9 และศักยภาพในการประยุกต์ใช้ในเชิงการเกษตร (PHAGE DISPLAYED ANTIBODY AGAINST *BRADYRHIZOBIUM YUANMINGENSE* STRAIN DOA9 AND ITS POTENTIAL APPLICATION IN AGRICULTURE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.พรรณลดา ติตตะบุตร, 108 หน้า.

Bradyrhizobium เป็นแบคทีเรียที่พบในดินชนิดหนึ่ง ที่มีความสามารถในการตรึงไนโตรเจน และอยู่อาศัยร่วมกับพืชตระกูลถั่วบางชนิดที่มีความจำเพาะเจาะจงกันได้ โดยทำหน้าที่ในการเปลี่ยนแก๊สไนโตรเจนในบรรยากาศไปเป็นแอมโมเนีย ซึ่งเป็นรูปแบบของปุ๋ยไนโตรเจนที่พืชสามารถนำไปใช้ได้โดยตรง *Bradyrhizobium* sp. สายพันธุ์ DOA9 ที่ใช้ในการศึกษาครั้งนี้ได้คัดแยกออกมาจากปมรากของต้นโสนหางไก่ (*Aeschynomene americana*) ในประเทศไทย ทั้งนี้จากการตรวจวิเคราะห์ลำดับนิวคลีโอไทด์โดยเทคนิค multilocus DNA sequencing ของยีน 16S rRNA และยีนพื้นฐานอื่น ๆ (ยีน *dnaK*, *recA*, และ *glnB*) พบว่าแบคทีเรียสายพันธุ์นี้จัดอยู่ในกลุ่ม *B. yuanmingense* และจากความสามารถของแบคทีเรียสายพันธุ์นี้ในการสร้างปมกับพืชตระกูลถั่วได้หลากหลายชนิด ดังนั้น DOA9 จึงเป็นแบคทีเรียสายพันธุ์ที่น่าสนใจในการนำไปประยุกต์ใช้ เพื่อเป็นหัวเชื้อปุ๋ยชีวภาพในทางการเกษตร พร้อมทั้งใช้ในการศึกษาด้านปฏิสัมพันธ์กับพืชชนิดต่าง ๆ ต่อไป

ในวิทยานิพนธ์นี้ได้ดำเนินการสร้างคลังของ recombinant scFV แอนติบอดี้ขึ้นจากม้ามของกระต่ายที่ถูกฉีดกระตุ้นภูมิคุ้มกันด้วยแบคทีเรีย สายพันธุ์ DOA9 จากนั้นทำการคัดเลือกหา scFV แอนติบอดี้จากคลังที่สร้างขึ้นนี้ พร้อมทั้งจากคลัง scFV แอนติบอดี้ที่สร้างจากมนุษย์ ที่สามารถจับกันแบบจำเพาะได้กับเซลล์เป้าหมาย DOA9 โดยใช้เทคนิค biopanning จากการทดลองสามารถคัดเลือก scFV phage ได้จำนวน 2 โคลน คือ RB8 และ RG9 จากคลังแอนติบอดี้ที่สร้างจากกระต่าย และอีกจำนวน 1 โคลน คือ RD6/2 จากคลังแอนติบอดี้ที่สร้างจากมนุษย์ ทั้งนี้ได้ทำการทดสอบยืนยันความจำเพาะเจาะจง ในการจับกันระหว่างแอนติเจน และแอนติบอดี้ที่คัดเลือกได้โดยเทคนิค Phage Enzyme Linked Immunosorbent Assay (Phage ELISA) โดยผลที่ได้พบว่าแอนติบอดี้จาก phage สามารถจับกับแบคทีเรียสายพันธุ์ DOA9 ทั้งที่อยู่ในรูปเซลล์อิสระ (ในอาหารเลี้ยงเชื้อ) และในรูปแบบ bacteroid ที่อยู่อาศัยในปมรากพืชได้อย่างจำเพาะเจาะจง และพบว่าแอนติบอดี้ที่คัดเลือกได้ ยังสามารถประยุกต์ใช้ได้กับการย้อมเซลล์แบคทีเรีย ด้วยเทคนิค immunofluorescence ซึ่งสามารถตรวจสอบได้ภายใต้กล้อง confocal microscope นอกจากนี้ยังได้ทดสอบการผลิต scFV แอนติบอดี้ในรูปแบบของแอนติบอดี้ที่ละลายได้ โดยวิธีการตัดต่อยีนไป

บนพลาสมิด pETb21 เพื่อให้แสดงออกในเซลล์ *Escherichia coli* HSM 174 จากนั้นแยกแอนติบอดี้ที่ได้ออกมาจากเซลล์โดยใช้ Ni-NTA ซึ่งทำให้ได้ปริมาณแอนติบอดี้บริสุทธิ์ที่มากเพียงพอ และยังคงมีคุณสมบัติในการจับกับแอนติเจนได้อย่างจำเพาะเจาะจงเช่นเดิม ผลการทดลองนี้แสดงให้เห็นถึงศักยภาพของการนำแอนติบอดี้ที่ได้ ไปประยุกต์ใช้ในการตรวจสอบไรโซเบียมชนิดนี้ในขั้นตอนการผลิตหัวเชื้อในห้องปฏิบัติการ และสามารถใช้ติดตามไรโซเบียมจากปมถั่วเมื่อนำไปใช้ในภาคสนามได้ การศึกษาครั้งนี้เป็นครั้งแรกของการคัดแยกแอนติบอดี recombinant scFV จากคลังแอนติบอดี้ที่สร้างจากกระต่าย และจากมนุษย์ ที่สามารถจับกับเชื้อแบคทีเรียกลุ่มที่มีความสามารถในการตรึงไนโตรเจนได้ ทำให้เป็นการกระตุ้นให้เกิดการศึกษาเกี่ยวกับแอนติบอดี้ recombinant scFV เพื่อพัฒนาวิธีการตรวจสอบเชื้อแบรดดี้ไรโซเบียมในทางการเกษตรที่ทำได้ง่ายและรวดเร็ว ตลอดจนใช้ในการพัฒนาการศึกษาเชิงลึกเกี่ยวกับปฏิสัมพันธ์ระหว่างพืชและจุลินทรีย์ต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ
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VU NGUYEN XUAN : PHAGE DISPLAYED ANTIBODY AGAINST *BRADYRHIZOBIUM YUANMINGENSE* STRAIN DOA9 AND ITS POTENTIAL APPLICATION IN AGRICULTURE. THESIS ADVISOR : ASST. PROF. PANLADA TITTABUTR, Ph.D., 108 PP.

PHAGE DISPLAY/SCFV/PHAGE DISPLAY ANTIBODY LIBRARY/ *BRADYRHIZOBIUM YUANMINGENSE* STRAIN DOA9

Bradyrhizobium is one of the soil bacteria that generally fix nitrogen in symbiosis with specific leguminous plants and converts nitrogen gas into ammonia, which is a form of nitrogen that plants can directly utilize as fertilizer. *Bradyrhizobium* sp. DOA9 is a bacterial strain originally isolated from the root nodules of *Aeschynomene americana* in Thailand. This strain was classified as *B. yuanmingense* based on a multilocus DNA sequence analysis of its 16S rRNA and housekeeping genes (*dnaK*, *recA*, and *glnB*). Due to its ability to be used with a broad range of hosts , DOA9 is an interesting strain that can also be used as multi-purposes inoculant for biofertilizer and has been investigated for its interaction with plants in other aspects.

In this thesis, the recombinant scFv antibody library was constructed from the spleen of a rabbit immunized with strain DOA9. This library (the immunized rabbit scFv antibody library) as well as the naïve human scFv antibody library were used for selecting specific antibodies which act against the target, strain DOA9 by the biopanning method. After biopanning, two scFv phage clones, RB8 and RG9 were selected from the immunized rabbit library. After sequencing, it was found that the two phage clones were identical. Similarly, after biopanning, one scFv phage clone, RD6/2 was

selected from the naïve human library. The specific binding of the three antibody clones was confirmed by Phage Enzyme Linked Immunosorbent Assay (Phage ELISA). The phage antibody could bind specifically to DOA9 both as a free-living (pure culture) and as a bacteroid inside the plant nodule stages. Moreover, specific immunofluorescence staining of both free-living bacteria and bacteroid inside the plant nodule could also be observed with a confocal microscope. Also, soluble scFv antibodies were produced by subcloning into a/the pETb21 vector and were expressed in *Escherichia coli* HSM 174. The soluble scFv antibody specimens were purified using Ni-NTA (Nickel-nitrilotriacetic acid). The purifications yielded an appropriate amount of adequate pure scFv's, and the antibody specificity was retained. These results indicate a potential application for verifying this bacterium during a production process or for monitoring this strain from a/the nodule after application in the field. This study also describes, for the first time, the isolation of a/the recombinant scFv antibody against N-fixing bacteria from an immunized rabbit library and also a naïve human library. The result encourages further investigation of a recombinant scFv antibody for the development of new immunoassays for more rapid and simpler detection of *Bradyrhizobium* in agriculture as well as for an in-depth analysis of plant-microbe interactions in the future.

School of Biotechnology

Academic Year 2015

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LIST OF ABBREVIATIONS

×*g*	=	times gravity
°C	=	degree Celsius
µg	=	microgram
µl	=	microliter
bp	=	base pair
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
et al.	=	et alia (and other)
g	=	gram
h	=	hour
l	=	liter
mg	=	milligram
min	=	minute
ml	=	mililiter
mM	=	millimolar
N	=	normality
ng	=	nanogram
PCR	=	polymerase chain reaction
ρmol	=	picomole
RNA	=	ribonucleic acid
rRNA	=	ribosomal ribonucleic acid

LIST OF ABBREVIATIONS (Continued)

UV	=	ultraviolet
v/v	=	volume per volume
w/v	=	weight per volume

CHAPTER I

INTRODUCTION

1.1 Background

In plant, nitrogen is a critical limiting element for plant growth and production thus it is applied as N-fertilizer to increase yield of important crops. However, the excess uses of nitrogen fertilizer have various adverse effects on soil, such as depletes water holding capacity, soil fertility and disparity in soil nutrients and water pollution. Therefore, a number of microorganisms (bacteria, fungi and algae) are considered as beneficial for agriculture and used as bio-fertilizers (Pedraza 2008). Rhizobium is one of biofertilizers that can nodulate specific leguminous plants and able to fix nitrogen from the atmosphere into the plants themselves. This process is called "Biological Nitrogen Fixation" (BNF). Nowadays, the BNF approach becomes a very attractive and promising alternative to expensive nitrogen chemical fertilizers. However, the number of rhizobia in the soil are low or containing higher number of ineffective strain. In order to take advantage of this association of bacteria and leguminous plants, it is often necessary to provide suitable legume inoculants to assure effective nodulation of leguminous crops. The application of these bacteria to seed or soil is called "inoculum" (Burton 1984).

The key success of having high quality legume inoculant is an effective quality control system. The rhizobial strain must be identified and the number of cell should be higher than the standard with no contamination (Olsen et al. 1996). Therefore, several methods are needed to validate the identity and quantity of specific

rhizobia in the inoculant. Nowadays, there are many methods for detection and monitoring of rhizobia in quality control procedures, such as microbiological techniques, immunological techniques and molecular techniques (Olsen et al. 1996). The immunological techniques used widely in quality control procedures to obtain information about rhizobial cultures and products. However, polyclonal antibody is very often to have cross-reactivity with other rhizobial strains within the same species. This cross-reactivity may also extend to other rhizobial biovars or species and sometimes even to members of other bacterial genera (Olsen et al. 1996). These cross-reactions are the main problem of using polyclonal antibody to detect and monitor specific rhizobium. The hybridoma technology which enabled a defined specificity of monoclonal antibodies (mAbs) has been introduced to produce mAbs in consistent quality and in large quantities in the laboratory (Kohler et al. 1975). Since then, mAbs have been favored as they can be produced in unlimited quantities to practically bind to any antigen and are more easily standardized. However, mAbs face difficulties in production. The monoclonal antibody producing technology is laborious, time consuming, and costly (Ahmad et al. 2012), thus it is difficult to be produced and applied in agriculture.

Comparing to all antibody - based methods, phage display technology offers an attractive way of producing antibodies against diverse antigens. The phage display technique allowed isolation of antibodies directly from diverse repertoires of antibody genes. These antibody genes are expressed on the surface of filamentous bacteriophage as fusion proteins (Smith 1985, McCafferty et al. 1990, Smith et al. 1997). Recently, the technology has been improved through recombinant DNA technology and antibody engineering whereby antibody genes can now be cloned and expressed successfully as a fragment in many expression systems such as, bacteria

(Skerra et al. 1988), mammalian cell and yeast (Ho et al. 2006), plant (Galeffi et al. 2006), and also insect cells (Choo et al. 2002). One advantage of this new technology is that they could retain the intact antigen binding site (paratope) while reducing the size of the antibody molecule. In addition, the combination of small antibody molecule together with the efficient microbial production systems can finally lead to the production of a homogenous protein in sufficient amounts for diagnostic and therapeutic purposes as well as in structural studies (Ahmad et al. 2012).

Bradyrhizobium sp. DOA9, a non-photosynthetic bradyrhizobial strain was isolated from *Aeschynomene americana* L. in Thailand. Previous study based on phenotypic characteristics and sequence analysis of 16sRNA and 3 housekeeping genes (*dnaK, recA, glnB*) confirming that this strain is closely related to *B. yuanmingense*. This strain has board host range and be able to nodulate several legumes and colonize rice plant (Noisangiam et al. 2012). Thus, this strain has great potential in inoculant production for bio-fertilizers.

Thus, it is interesting to develop antibody specific to *Bradyrhizobium* sp. DOA9 by using engineering antibody through phage display technique to reduce the problem of cross-reaction occurring from polyclonal antibody or difficulty in monoclonal antibody production.

1.2 Research objectives

To construct a recombinant single-chain variable fragment antibody (scFv) against nitrogen fixing bacteria *Bradyrhizobium* sp. DOA9 for agricultural application.

Specific objectives for this thesis are:

1. To construct a phage displayed single chain fragment variable (scFv) antibodies from rabbit immunized with nitrogen fixing bacteria *Bradyrhizobium* sp. DOA9.

2. To affinity select phage displayed scFv antibodies which are specific to *Bradyrhizobium* sp. DOA9 by bio-panning from naïve human antibody and immunized rabbit antibody constructed from step 1.

3. To verify the selected single chain fragment variable (scFv) for the detection of *Bradyrhizobium* sp. DOA9 in different agricultural processes.

CHAPTER II

LITERATURE REVIEWS

2.1 Biological Nitrogen Fixation (BNF)

In nature, some prokaryotes can convert atmospheric nitrogen into a form that plants can use. They utilize the nitrogenase enzyme to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). This process is called biological nitrogen fixation (BNF). They consist of aquatic organisms, such as cyanobacteria, free-living soil bacteria, such as *Azotobacter*, bacteria that form associative relationships with plants, such as *Azospirillum,* and most importantly, bacteria Rhizobia, such as *Rhizobium* and *Bradyrhizobium* that form symbioses with legumes and other plants (Sessitscha et al. 2002).

The enzyme nitrogenase catalyzes the breaking of hydrogen bond and the addition of three hydrogen atoms to each nitrogen atom. Microorganisms that fix nitrogen require 16 moles of adenosine triphosphate (ATP) to reduce each mole of nitrogen. They obtain this energy by oxidizing organic molecules. Non-photosynthetic free-living microorganisms must obtain these molecules from other organisms, while photosynthetic microorganisms, such as cyanobacteria, use sugars produced by photosynthesis. The symbiotic nitrogen-fixing microorganisms obtain these compounds from their host plants (Bhattacharjee et al. 2008).

2.1.1 Rhizobia

Rhizobia are the general name given to a diverse group of soil bacteria which are able to symbiosis with leguminous plants. They are gram-negative chemoheterotrophic organotroph bacilli that live freely in the soil. Rhizobia could form the specialized organs, called nodules, on roots or stems of leguminous hosts. The nodule inside bacteria could convert atmospheric nitrogen into ammonia, providing the nitrogen requirements of both rhizobia and their host plants. In feedback way, the plant feeds sugars, proteins, and oxygen for the bacteria (Sessitscha et al. 2002).

In the past, rhizobia were divided into two groups depending on growth rate: *Rhizobium* and *Bradyrhizobium*. The firstly genus, *Rhizobium* is the fast-growing acid producers that develop pronounced turbidity in liquid media within 2-3 days and have a mean doubling time of 2-4 h. The cells are motile by two to six peritrichous flagella. They can grow on a wide range of carbohydrates, but usually grow best on glucose, mannitol, or sucrose. This group is generally effective on temperate legumes. The second genus, *Bradyrhizobium*, is emerged as the genus of the slow-growing and alkaline-producing rhizobial strains. They are the slow-growing, alkali-producing rhizobia, collectively known as bradyrhizobia. They require 3-5 days to produce moderate turbidity in liquid media and have a mean doubling time of 6-8 h. Most strains in this group grow best with pentoses as their carbon source. The cells are motile by a single polar or subpolar flagellum. A large genera of tropical legume species are nodulated by bradyrhizobia (Somasegaran et al. 1994).

The classification and taxonomy of rhizobia is becoming increasingly complex and is revised periodically because of new findings that propose new genera and new species. DNA homology values, guanine-cytosine (GC) content, sequence

homologies of multiple loci (such as small subunit ribosomal RNA gene (16S rDNA), house-keeping genes and symbiotic genes), locations of symbiotic genes and phenotypic characteristics provide more and deeper information for the classification of rhizobia (Pongsilp 2012).

To date, rhizobia have been classified into six genera, including *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Allorhizobium*, *Sinorhizobium*, and *Rhizobium* (Martinez-Romero et al. 2000, Teamroong et al. 2006, Pongsilp 2012). The current genera of Rhizobiaceae are summarized in Table 2.1

2.1.2 Rhizobial antigens

Antigens of rhizobia can be categorized into somatic, flagella, and capsular, depending on their derivation. Somatic antigens are closely related to the rhizobial cell wall and are usually designated by the letter O. Some somatic antigens may be tightly bound to the cell wall, in which case they are not removed by washing of the cells. Therefore, these antigens are only detected when whole cells of rhizobia react with the antibody, as in agglutination or immunofluorescence. The somatic antigens that are soluble and easily removed by washing are detected by precipitation in gel. Somatic antigens are also heat stable. They are the most specific of the three groups of antigens.

The precipitating "internal antigens" are more widely shared and taxonomically significant. These are released from cells having fragile or broken walls. Because internal antigens are widely cross-reactive within and between species, they require recognition and interpretation in gel immune-diffusion.

Table 2.1 Current taxonomy of rhizobia (Martinez-Romero et al. 2000).

Genus	Species	Host
Allorhizobium	*A. undicola*	*Nepunia natans*
Azorhizobium	*A. caulinodans*	*Sesbania rostrata*
Bradyrhizobium	*B. elkanii*	*Glycine max*
	B. japonicum	*G. max*
	B. liaoningense	*G. max*
Mesorhizobium	*M. amorphae*	*Amorpha fructicosa*
	M. cicero	*Cicer arietinum*
	M. huakuii	*Astragalus*
	M. loti	*Lotus japonicas*
	M. mediterraneum	*C. arietinum*
	M. plurifarium	*Acacia, Leucaena*
	M. tianshanse	*Glycyrrhiza, Sophora, Glycine* and others
Rhizobium	*R. etli*	*Phaseolus vulgaris, Mimosa affinis*
	R. galegae	*Galega*
	R. gallicum	*P. vulgaris*
	R. giardinii	*P. vulgaris*
	R. hainanense	*Stylosanthes, Centrocema, Desmodium,*
	R. huantlense	*Tephrosia*
	R. leguminosarum	*S. herbacea*
	R. mongolense	*Vicia, Trifolium*
	R. tropici	*Medicago ruthenica, P. vulgaris* *P. vulgaris, Leucaena*
Sinorhizobium	*S. arboris*	*A. senegal, Prosopis chilensi*
	S. fredii	*G. max*
	S. kostiense	*A. senegal, P. chilensis*
	S. medicae	*Medicago* spp.
	S. meliloti	*M. sativa*
	S. saheli	*Sesbania*
	S. terangae	*Sesbania, Acacia*
	S. xinjiangense	*G. max*

The flagella of the rhizobia are also antigenic and appropriately called flagella or H antigens. They are heat labile and are commonly detected by agglutination or immunofluorescence test. The extracellular antigens are surface

antigens and are found outside the cell itself. They are usually designated by the letter K.

2.2 The methods to detect and monitoring of *Rhizobia*

Rhizobia that have dramatic differences in such important traits as host specificity, invasiveness, and effectiveness are indistinguishable from each other under the microscope. However, the detection and monitoring of rhizobial strain after introducing to the field are difficult (Somasegaran and Hoben 1994).

2.2.1 The microbiological techniques

Many morphological characteristics and biochemical and metabolic tests are frequently used to differentiate among rhizobial species. These tests include vitamin requirements, salt, acid and alkali tolerance, carbohydrate utilization, and resistance to antibiotics. Numerical analysis employs a large range of biochemical and metabolic tests to differentiate among rhizobial species. The results obtained from numerical analysis support the proposal of several novel species of rhizobia. Rhizobial species vary in their enzymatic production and several enzymes are found to be necessary for the symbiotic effectiveness. Carbohydrate utilization properties are of taxonomic significance (Somasegaran and Hoben 1994).

The possibility of resistance to low levels of antibiotics also can be used for rhizobial strain characterization and identification. When high-density inoculant of a rhizobial strain is inoculated into media containing an antibiotic, a few cells may exhibit resistance as a result of spontaneous genetic changes or mutations. The resistance of a rhizobial strain to a particular antibiotic is a useful marker. If the mutant strain is used to inoculate a legume, then nodules occupied by that strain may

be identified by plating nodule isolates on media containing the respective antibiotic. The mutant rhizobial strain will grow on the antibiotic media and other bacteria will be suppressed. Streptomycin resistance is frequently used as a marker for rhizobia. Mutants resistant to this aminoglycoside are stable, have a low incidence of cross-resistance, and infrequently lose their symbiotic capacity. Besides streptomycin, spectinomycin and rifampicin have also been used (Olsen et al. 1996).

2.2.2 The immunological techniques

When bacteria (including rhizobia) are injected into a mammal, the animal produces antibodies which will bind antigens on the surface of the bacteria. As a result of antigen injections, complex immunological reactions result in the animal producing special proteins called globular antibodies (immunoglobulins). The study of the reactions of the immune serum with the antigens outside the animal is known as serology. If the surface antigens on the rhizobia are relatively unique and unshared by other microorganisms the binding of the antibodies can be used to both detect and identify the target rhizobia. Antigen-antibody reactions are highly specific. The antibody reacts only with the antigen that elicited its formation. A variety of method based on antibody-antigen reactions and which are useful in the detection of rhizobial broth or inoculants have been developed. In rhizobial, both cultured cells and nodule antigens (bacteroids) are used for strain identification (Olsen et al. 1983, Somasegaran and Hoben 1994, Olsen et al. 1996).

Nowadays, several methods have been applied to study the phenotypic diversity of rhizobia, particularly numerical analysis, enzyme pattern and serological study. Serological techniques are the most specific methods for identifying rhizobia based on natural marker characteristics. Phenotypic characterization by serotyping has

been used widely to study rhizobial populations in different geographical origins. Rhizobia in different serogroups have been found predominant among field populations (Pongsilp 2012).

2.2.2.1 Agglutination

The process in which the antigens are linked together by their corresponding antibodies is called agglutination. The linked antigens may be microscopically or macroscopically visible as clumps, agglutinates or aggregates. The agglutination reaction depends on a firm structural relationship between an exposed bacterial antigen and the antibody. If the antibodies do not bind to the cells, no agglutination occurs. Agglutination is commonly used as a method of identifying specific bacterial antigens, and in turn, the identity of such bacteria. The agglutination test was one of the first methods to be applied to serological investigations of rhizobial bacteria. It is among the simplest of serological techniques to use and it has been widely applied in many taxonomic and ecologic investigations (Somasegaran and Hoben 1994).

Advantages: the agglutination reaction is the simplest of all immunological procedures and involves only primary antisera. No secondary antibodies or signal producing labels are needed. The technique is fast and does not require specialized equipment and skill.

Limitations: The cross-reactivity between rhizobia is common with non-adsorbed primary antisera. The agglutination test is not as sensitive as other immunological tests and does not demonstrate cell viability (Olsen et al. 1996).

2.2.2.2 Precipitation

In recent years, the precipitation tests of somatic antigens have been used widely for rhizobia. The precipitation reaction occurs when certain soluble antigens are brought into contact with the corresponding antibody. Precipitation differs from agglutination in that the precipitating antigens are not whole bacterial cells (cellular), but are proteins or polysaccharide molecules in solution (Somasegaran and Hoben 1994).

2.2.2.3 Immunodiffusion

The somatic antigens of many rhizobium strains diffuse slowly in the agar gels; they yield either no precipitin bands or only weak bands close to the antigen well since the location of bands is dependent upon the relative concentrations of diffusable antigens and antibodies (Somasegaran and Hoben 1994).

2.2.2.4 Immunofluorescence (IF)

One of the most sensitive of the serological methods available to study rhizobia is the fluorescent antibody (FA) technique. It allows for the visualization and investigation of the antigens of individual cells with the fluorescent microscope and requires only small quantities of both antigen and antibody. In contrast both agglutination and immunodiffusion require large amount of antigen and antisera to give a visible reaction.

Certain chemical dyes such as fluorescein isothiocyanate (FITC) and lissamine rhodamine have the property of fluorescing when excited by near UV light. Rhizobial antibodies developed in rabbits can be conjugated to these fluorescing chemical dyes or fluorochromes. In work with rhizobia, the chemical dye commonly

used for labeling the specific antibody is FITC, which has an apple-green fluorescence upon irradiation with blue light.

There are two types of fluorescent antibody techniques, namely the direct and indirect immunofluorescence. In the direct method, the specific antiserum is conjugated and is used as a stain in the procedure. This is different from the indirect method, where the unconjugated (unlabeled) specific or primary antibody is first reacted with the antigen smear, and after sufficient time is allowed for antigen-antibody reaction, the smear is then washed free of excess antiserum. This step is followed by staining with the FITC-labeled secondary antibody.

In serological work with rhizobia, the specific or primary antibody against the rhizobial strain is most often developed in rabbits. The secondary antibody is developed by immunization of goats or sheep with purified rabbit immunoglobulins from a previously unimmunized rabbit. Thus, the rabbit immunoglobulin serves as an antigen for immunization of the goat or sheep. Therefore, the antibody produced in the goat or sheep will not only react with the rabbit antiserum, but will also react with rhizobial antigen with specific unlabeled rabbit antibody attached when the indirect procedure is employed. Though the results are the same, the indirect method is considered more sensitive. The indirect method requires the labeling of only the immune serum from the goat or sheep, and involves two reaction steps; the indirect method is also known to give more nonspecific staining reactions. In the direct method, each rabbit antiserum developed against each rhizobial strain must be conjugated (Somasegaran and Hoben 1994, Olsen et al. 1996).

Fluorescent antibody (FA) has been applied widely in working with rhizobia, such as, used to identify strains of rhizobia, to identify the nodule-bacteria, to detect doubly infected nodules, to study rhizobium in soil, to study

population dynamics of *R. japonicum* in the rhizosphere, and to make quantitative studies of rhizobium in soil. The FA test is rapid and simple but requires fluorescent microscopy equipment and special skill (Somasegaran and Hoben 1994).

2.2.2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is one of the several enzyme immunoassays used in detecting antigens and antibodies. Basically, in an enzyme immunoassay, either the antibody or antigen is tagged with an enzyme (e.g., alkaline phosphatase). After completing the assay, the presence or absence of the enzyme-labeled component is detected by the addition of an appropriate substrate (e.g., paranitrophenylphosphate) resulting in a colored product.

In direct ELISA, the specific antibody (Abl) developed for a particular strain of rhizobia is immobilized in the wells of the plate. Excess unreacted Abl is washed off. The rhizobial antigen is then added to the Ab1-coated wells. After an incubation period, excess unreacted antigen is removed by washing. This is followed by the addition of an enzyme-Abl conjugate, which binds to its specific antigen. Excess enzyme Abl is washed off. The substrate is then added and the reaction is stopped following incubation; the colored product is measured colorimetrically by ELISA reader machine.

In indirect ELISA, which is more popular with rhizobial workers, the antigen is immobilized first in the wells. This is followed by the addition of Abl, incubation, and washing. The next reactant added is enzyme-Ab2 conjugate. Ab2 is usually sheep or goat antibody against Ab1. The enzyme-Ab2 conjugate specifically binds to Ab1. After addition of the substrate, the reaction is completed as with direct

ELISA. In both ELISA approaches, a 96-well plastic microtiter plate (solid support) is used to immobilize the antigen or antibody.

The advantages of ELISA are requirement very small amounts of antiserum and no microscopic equipment is necessary. But, ELISA requires a purified antigen preparation, either from culture or from a root-nodule (Somasegaran and Hoben 1994, Olsen et al. 1996).

2.2.2.6 Membrane immunoblot

The membrane immunoblot procedure is another enzyme immunoassay that has been developed to detect antigen or antibodies (proteins) immobilized (bound) onto a membrane support. This technique has been applied in inoculant quality control and ecological studies of rhizobia. The rhizobial cells (antigens) are blotted or applied onto membranes made of nitrocellulose or nylon. After incubating, the membrane-bound antigens with the homologous antibody (Abl) solution, and washing to remove excess unbound Abl, the membrane is immersed in a solution containing enzyme Ab2. As with ELISA, Ab2 is usually sheep or goat antibody against Ab1. Ab2 has been conjugated with alkaline phosphatase enzyme and binds specifically to Ab1. The assay is completed by the addition of substrate reagents. These reagents are a mixture of 5-bromo-4-chloro-3- indolyl-phosphate (BClP) and Nitro Blue Tetrazolium (NBT).

This technique is not complex and requires no specialized equipment. However, like all immunological techniques for identifying rhizobia, the results of the spot blot test are only as good as the specificity of the anti-rhizobial antisera used. Also, the test does not demonstrate cell viability (Somasegaran and Hoben 1994, Olsen et al. 1996).

2.2.3 The DNA-based techniques

Nowadays, the DNA-based techniques have been widely used for evaluating the genetic diversity of microorganisms. For rhizobial, the several techniques have been employed to examine genotypic diversity of rhizobial populations and to discriminate among rhizobial strains. These techniques include random amplified polymorphic DNA (RAPD), two-primers RAPD (TP-RAPD), repetitive sequence based PCR (rep-PCR) and amplified fragment length polymorphism (AFLP). The 3 main techniques of rep-PCR are enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR and BOX-PCR. While RAPD uses a single primer to amplify the segments of DNA randomly throughout the genome, rep-PCR uses pairs of primers (for ERIC-and REP-PCR) or a single primer (for BOX-PCR) to amplify the intervals between conserved repeated sequences present in genome. In AFLP, total genomic DNA is digested and then ligated to oligonucleotide adapters. A pair of specific primer is used to amplify the product from restriction. RAPD, rep-PCR and AFLP are suitable for distinguishing strains at species or below levels but they are less valuable for taxonomic purpose. TP-RAPD has been developed for taxonomic purpose as the patterns of strains in the same species have been found to be identical. The TP-RAPD patterns supported the proposal of novel species of rhizobia (Pongsilp 2012).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is used in determining the genetic relationships based upon PCR and restriction analysis. Specific genes, such as small subunit ribosomal RNA gene (16S rDNA), large subunit ribosomal RNA gene (23S rDNA), 16S-23S rRNA intergenic spacer (IGS) and symbiotic genes have been used in PCR-RFLP. The PCR-RFLP profile is used to estimate the genetic diversity of microorganisms. The PCR-RFLP

method has been used successfully in the differentiation of rhizobial species (Pongsilp 2012).

Restriction fragment length polymorphism (RFLP) also has been used to examine the genotypic diversity of bacteria. The technique is based on restriction analysis and hybridization, resulting in the fingerprint patterns. For rhizobia, symbiotic genes have been frequently used as probes for hybridization. Based on the same approach, the insertion sequences (ISs) can be used as probes, resulting in the patterns termed as "IS fingerprints". The use of ISs as probes for hybridization can provide high-resolution fingerprints of rhizobial strains. The ISs have been found to be abundant in rhizobia. The distribution of ISs varies widely in both IS type and copy number. Several ISs are specific to rhizobial species (Pongsilp 2012).

The identification of bacteria based on phenotypic characteristics is generally not accurate because several species are very difficult to be distinguished phenotypically. F rhizobia, the current classification is mainly based on DNA sequences [especially a DNA sequence encoding small subunit ribosomal RNA (16S rDNA)], DNA homologies, phylogenetic relationships and the locations of symbiotic genes. The 16S rDNA is very useful for estimating the evolutionary relationships and identifying bacteria. For bacterial identification, 16S rDNA sequencing is particularly important in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria and culture-negative infections. The most dramatic progress in microbial phylogeny and taxonomy is based on sequence analysis of 16S rDNA. These sequences mainly support the proposal of novel genera and species of rhizobia. In some cases, several genera are identical in 16S rDNA sequence analysis. The other regions, such as large subunit ribosomal RNA gene (23S rDNA) as well as intergenic spacer between 16S and 23S rRNA sequences (16S-23S

IGS), are suitable alternatives for classification and identification purposes. Multilocus sequence analysis (MLSA), which employs a set of nucleotide sequences including 16S rDNA, house-keeping genes and symbiotic genes, has the greater potential for rhizobial classification. Currently, sequence analysis is the most promising and useful method for identification of rhizobial genera (Pongsilp 2012).

2.3 Phage display technology as possible tool to detect and monitor rhizobia

2.3.1 Recombinant antibody

An antibody (Ab), also known as an immunoglobulin (Ig), is a large Y-shape protein produced by plasma cells that is used by the immune system to identify and neutralize foreign objects such as bacteria and viruses. The antibody recognizes a unique part of the foreign target, called an antigen. Each tip of the "Y" of an antibody contains a paratope (a structure analogous to a lock) that is specific for one particular epitope (similarly analogous to a key) on an antigen, allowing these two structures to bind together with precision. Using this binding mechanism, an antibody can tag a microbe or an infected cell for attack by other parts of the immune system, or can neutralize its target directly (for example, by blocking a part of a microbe that is essential for its invasion and survival). The production of antibodies is the main function of the animal immune system.

Immunoglobulin G (IgG) is an antibody isotype. It is a protein complex composed of four peptide chains-two identical heavy chains and two identical light chains arranged in a Y-shape typical of antibody monomers. Each IgG has two antigen binding sites. Immunoglobulin G (IgG) is a heterotetrameric molecule

consisting of two heavy and two light chains, respectively, which are connected via disulfide bonds. Heavy and light chains (HC and LC) also contain intramolecular disulfide bonds for stabilization (Edelman 1973). IgG is the most abundant antibody isotype found in the circulation, approximately 75% of serum immunoglobulins in humans.

These structural properties require a sophisticated folding apparatus as well as an oxidizing environment for the generation of disulfide bonds. Consequently, many traditionally expression hosts do not provide these mechanisms for efficient production of IgGs. Therefore, smaller antibody fragments have been developed which combine easier production with full antigen binding capacity of an IgG (Frenzel et al. 2013).

Recently, the technology has been improved by recombinant DNA technology and antibody engineering whereby antibody genes can now be cloned and expressed successfully as a fragment in bacteria (Skerra and Plückthun 1988), on mammalian cell and yeast (Ho et al. 2006), plant (Galeffi et al. 2006) and also insect cells (Choo et al. 2002). One advantage of this new technology is that they could retain the intact antigen binding site (paratope) while reducing the size of the antibody molecule. In comparison to the parental antibody, these minimized antibodies have several advantages in clinical practices including better tumor penetration, more rapid blood clearance, and lower memory times in non-target tissue. It also could lead to the expression of the functional antibody and their fusion in bacteria and also allow their display on a filamentous phage. In addition, the combination of small antibody molecule together with the efficient microbial production systems can finally lead to the production of a homogenous protein in sufficient amounts for diagnostic and therapeutic purposes as well as in structural studies (Ahmad et al. 2012).

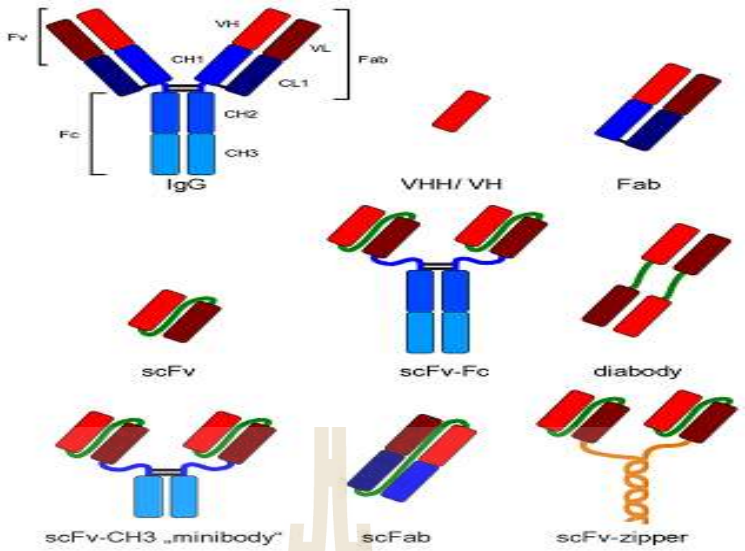

Figure 2.1 Recombinant antibody formats for different applications compared to IgG. Red and dark red: variable regions; blue: constant regions; green: artificial peptide linkers; yellow: dHLX represents amphiphatic helices used for dimerization of scFv fragments (Frenzel et al. 2013).

The recombinant antibody libraries have some type of libraries, naive antibody libraries, immune antibody libraries, synthetic antibody libraries, semi-synthetic antibody libraries.

2.3.1.1 Naive antibody libraries

Non-immune (or naïve) libraries are derived from natural, unimmunized, rearranged V-genes of animal or human. (Pansri et al. 2009). Key advantages of single-pot repertoires include: (i) isolation of human antibodies to self, non-immunogenic or toxic antigens; (ii) a single library can be used for all antigens; (iii) short time needed for antibody generation (2-4 rounds of selection in two weeks); and (iv) direct isolation of high affinity antibodies when very large repertoires are used. Disadvantages of naïve libraries are: (i) low affinity of antibodies isolated from small sized libraries; (ii) the time needed to construct large libraries, and (iii) content

and quality of the library are influenced by the unequal expression of the V-genes repertoire, unknown history of the B-cell donor, and potential limited diversity of the IgM repertoire (Azzazy et al. 2002).

2.3.1.2 Immune antibody libraries

In this library, V-genes are derived from the IgG mRNA of B-cells from an immunized animal. Immune libraries are advantageous in that antigen-specific and affinity-matured clones are enriched (Weisser et al. 2009). However, disadvantages of immune libraries include: (i) long time required for animal immunization, (ii) lack of immune response to self or toxic antigens, (iii) the unpredictability of the immune response to the antigen of interest, (iv) a new antibody library must be constructed for each antigen (this increases the total time of the procedure by 1-3 months), and (v) restrictions in generating human antibodies (Azzazy and Highsmith 2002).

2.3.1.3 Synthetic antibody libraries

Synthetic repertoires are libraries in which the antibodies are built artificially by *in vitro* assembly of V, D and J gene segments. Artificial V-genes assembly can include introducing a predetermined level of randomization of CDR regions into germline V-gene segments (Azzazy and Highsmith 2002, Zhou et al. 2011).

2.3.1.4 Semi-synthetic antibody libraries

Semi-synthetic libraries combine elements of natural and synthetic diversity and often are engineered to increase natural diversity while maintaining a certain level of synthetically introduced functional diversity (Zhou et al. 2011).

2.3.2 Single chain fragment variable (scFv)

The variable fragment is the smallest unit of immunoglobulin molecule with function in antigen-binding activities. An antibody in scFv format consists of variable regions of heavy (VH) and light (VL) chains, which are joined together by a flexible peptide linker that can be easily expressed in functional form in *E. coli*, allowing protein engineering to improve the properties of scFv such as increase of affinity and alteration of specificity (Griffiths et al. 1998).

The length of the flexible DNA linker used to link both of the V domains is critical in yielding the correct folding of the polypeptide chain. Previously, it has been estimated that the peptide linker must span 3.5 nm (35 Å) between the carboxyl terminus of the variable domain and the amino terminus of the other domain without affecting the ability of the domains to fold and form an intact antigen-binding site. In addition to the linker peptides designed *de novo*, peptide sequences derived from known protein structure have been applied to provide a compatible length and conformational in bridging the variable domains of a without serious steric interference. Apart from the length of the linker, their amino acid composition also plays an important role in the design of a viable linker peptide. They must have a hydrophilic sequence in order to avoid intercalation of the peptide within or between the variable domains throughout the protein folding. Nowadays, the most extensively used designs have sequences comprising stretches of Gly and Ser residues which meant for flexibility and or together with the charged residues such as Glu and Lys interspersed to enhance the solubility (Ahmad et al. 2012).

The scFv antibodies have been constructed mainly from hybridoma (Singh et al. 2010), spleen cells from immunized mice (Hayhurst et al. 2003, Wang et al. 2006) and B lymphocytes from human (Reiche et al. 2002, Pansri et al. 2009). The

scFv is a non-covalent heterodimer comprised of the VH and VL domains (Skerra and Plückthun 1988). For producing, mRNA is first isolated from hybridoma (or also from the spleen, lymph cells, and bone morrow) followed by reverse transcribed into cDNA to serve as a template for antibody genes amplification (PCR). With this method, large libraries with a diverse range of antibody VH and VL genes could be created (Pansri et al. 2009). In the scFv construction, most of them are constructed in a VH-linker-VL orientation (Ahmad et al. 2012). One of the most popular methods used is through PCR assembly which was first described by Horton et al. (1990). In this method, it allows the V domains of antibody to be cloned without any prior information about the nucleic acid as well as amino acid sequence of the particular antibody. Moreover, the V domains of antibody can be combined by *in vitro* recombination directly after the PCR of VH and VL genes into plasmid (Chaudhary et al. 1990) or phagemid (Ahmad et al. 2012).

Numerous scFv have been constructed against hapten (Kobayashi et al. 2005), protein (Dai et al. 2003), carbohydrate (Ravn et al. 2004, Sakai et al. 2007), receptor (Galeffi et al. 2006), tumor antigen (Shadidi et al. 2001, He et al. 2002), and viruses (Griep et al. 2000, Hu et al. 2005, Saldarelli et al. 2005). All these scFv have good potential for use in many fields such as medical therapies and diagnostic applications.

Nowadays, scFv have been successfully isolated and displayed as fragments in various expression systems such as mammalian cell and yeast (Ho et al. 2006), plant (Galeffi et al. 2006), and also insect cells (Choo et al. 2002). The scFv antibody can be expressed as correctly folded and directly active proteins or as aggregates requiring *in vitro* refolding to become active. Depending on the expression system, it varies in their ability to fold and secrete the scFv proteins. There are some

general regulations to consider on the design of vectors and expression system used with the different hosts and each of this host has advantages and disadvantages for the production of active antibody (Verma et al. 1998). Nevertheless, the bacterial expression system is most often applied for the production of scFv antibody fragments compared to the various expression strategies available (Frenzel et al. 2013).

2.3.3 Page display

2.3.3.1 Principle of phage display

Phage display was first described by George P. Smith in 1985, when he demonstrated the display of peptides on filamentous phage by fusing the peptide of interest on to gene III of filamentous phage (Smith 1985). Later, the technique was taken further by Greg Winter and John McCafferty at the Laboratory of Molecular Biology in Cambridge, UK, and Richard Lerner and Carlos F. Barbas at The Scripps Research institute, US, who independently used phage display to build large libraries of fully human antibody sequences. They have successfully demonstrated that a scFv fragment can be displayed on the phage surfaces as a functional protein which retains an active antigen-binding domain capability. This work laid the foundation for the development of human antibody based drugs (McCafferty et al. 1990). Therefore, this technology could allow rare clones to be screened and isolated from a large population of phage using any desirable antigen (Ahmad et al. 2012). Phage display is a laboratory platform that facilitates the study of protein to protein, protein to peptide, and protein to DNA interactions. Since its invention in 1985, phage display has been successfully applied to many different fields of research including immunology, cancer research, drug discovery, epitope mapping, protein-protein interactions, plant sciences, and infectious diseases,

targeting a broad cross-section of protein families. It has also been used to identify small peptide ligands and antibodies inhibiting the function of targeted receptors for a wide range of applications (Huang et al. 2012). Phage display is now playing a significant role for the discovery of peptides and antibodies that may serve as novel therapeutics (Nelson et al. 2010, Fjell et al. 2012).

Phage display involves the expression of peptides, proteins, or antibody fragment on the surface of filamentous bacteriophage. The interested DNA sequence is inserted into a location in the phage genome and fused to a gene encoding a phage coat protein. The interested protein is expressed or displayed on the surface of the phage particle, fused to one of the phage coat proteins. The phenotype of the expressed protein is thus linked to its genotype, which is present in the genome of the phage. Using recombinant DNA technology collections of billions of peptides, protein variants, gene fragments or cDNA-encoded proteins presented on phage can be constructed and surveyed for specific affinity and activity (Bratkovic 2010).

2.3.3.2 Biology of the filamentous bacteriophage

Although T4, T7, and λ phage have been used for phage display, the most commonly used phages are M13 and fd filamentous phage because they do not lyse infected bacteria during their life cycle (Huang et al. 2012).

Filamentous phages are a large family of bacterial viruses that infect many gram-negative bacteria by using the bacterial pili as a receptor. The most information about filamentous phages derives from those that infect *E. coli*: fl/M13/fd. They are usually referred to collectively, as Ff phages. These infect *E. coli* containing the F conjugative plasmid. The genomes of above three bacteriophages have been completely sequenced and are 98% homologous (Russel et al. 2004).

Structure of the phage particle: The native particle of filamentous phages is a thin, cylindrical shape, approximately 7 nm wide and 900nm in length. They have a single-stranded DNA genome (6,407base pairs in length) which encodes 11 genes (Carmen et al. 2002). The gene products are listed in Table 2.2

Table 2.2 Genes and gene products of fl bacteriophage (Webster 1996).

Gene	Amino acid	Function
I	Assembly	348
II	DNA replication	410
III	Minor capsid protein	406
IV	Assembly	405
V	Binding of ssDNA	87
VI	Minor capsid protein	112
VII	Minor capsid protein	33
VIII	Major capsid protein	50
IX	Minor capsid protein	32
X	DNA replication	111
XI	Assembly	108

The genes are grouped in the genome according to their functions in the life cycle of the bacteriophage (Figure. 2). There are three groups of genes (Webster 1996). The first group encodes proteins required for DNA replication. They consist of three genes (II, V, X). The second group encodes the proteins which make up the capsid. They consist of five genes (III, VI, VII, VIII and IX). The third group encodes three proteins related in the membrane assembly of the phage. They consist of three genes (I, IX, XI)

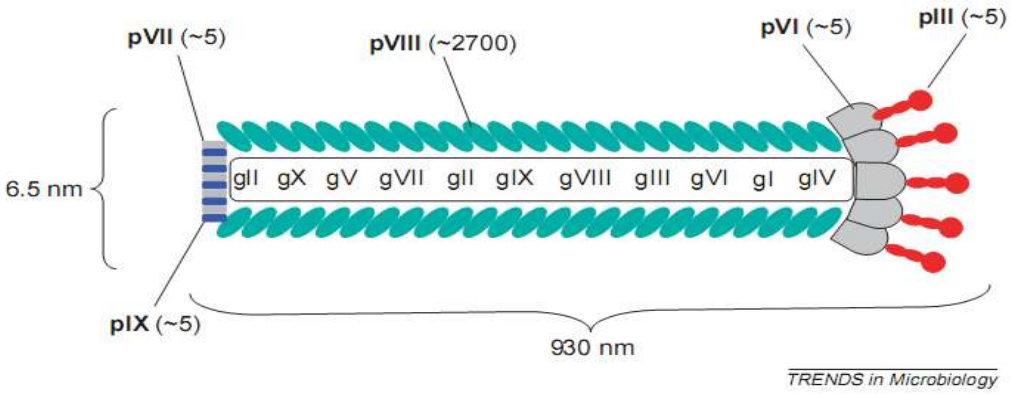

Figure 2.2 The schematic structure of filamentous bacteriophage, the copy number of each protein is shown in brackets (Mullen et al. 2006).

Life cycle: The stages of a phage life cycle are infection, replication of the viral genome, assembly of new viral particles, and then release of the progeny particles from the host. During phage infection, the pIII end of the phage attaches to the F pilus of male *E. coli*. Then, the pVIII major capsid proteins and other capsid proteins integrate into the inner bacterial membrane. The phage ssDNA is translocated into the cytoplasm of bacteria. This process requires the presence of the bacterial TolQRA protein (Karlsson et al. 2003). Mutations in any one of these genes block the uptake of the phage DNA into the cytoplasm. After that, the circular single-stranded viral DNA (plus strand) is converted to a double-stranded replicative form (RF) using the host cell DNA replication mechanism. The RF is then used to express all of the viral genes. The viral gene II product nicks the plus strand of the RF to initiate replication. In the initial stages of viral infection the newly synthesized plus strands of circular single-stranded DNA are converted to the RF which is used for protein synthesis and DNA replication. In the latter stages the synthesized single-stranded DNA is bound by the viral gene V protein (gpV-DNA) for viral assembly. The five viral structural proteins are synthesized and inserted into the inner membrane of the

host cell. The phage-assembly process involves both the gene I protein and the gene IV protein which form a pore in the membrane of the host cell from which the viral particle is released. The gene VII proteins and the gene IX proteins are assembled first. The gene V protein in the gpV-DNA complex is replaced by gpVIII coat as the phage is extruded from the host cell. Finally, the gene VI protein is assembled followed by gpIII, which terminates phage assembly (Webster 1996, Adda et al. 2002).

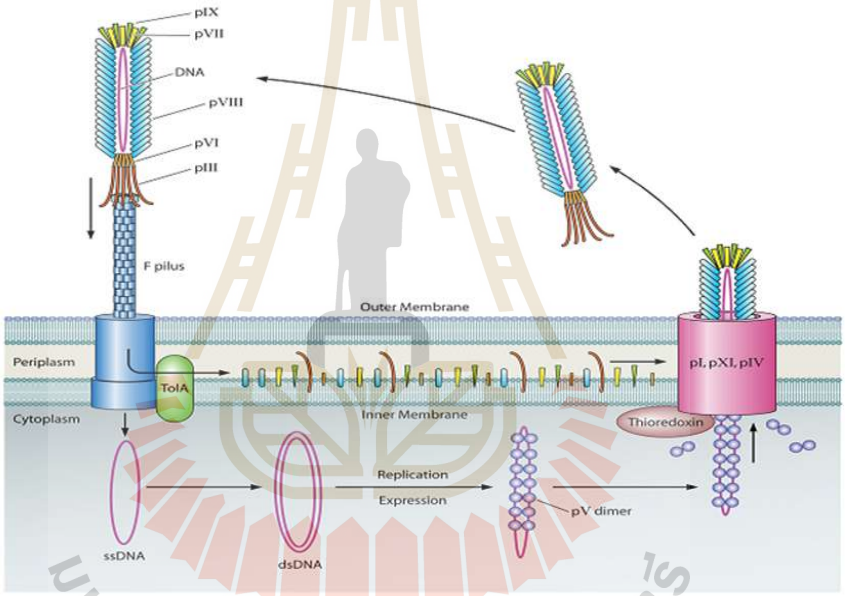

Figure 2.3 Life cycle of filamentous phage. Filamentous phage binds to the F pilus of a host bacteria cell thorough pIII. Then the host TolA protein starts to depolymerize the phage coat, which remain in the inner membrane for recycling. The ssDNA of the phage enters into the cytoplasma, converts into dsDNA and starts replication and expression using host enzymes, ssDNA and coated pV protein dimers form the precursors of the phage. Then pV is replaced by pIII in the chanel formed by pI, pXI, pIV and the host thioredoxin; in the meantime, mature phage particles are assemblyed and released (Russel et al. 2004, Huang et al. 2012).

2.3.3.3 Phage display vectors and types of phage display systems

Phagemids are Ff-phage-derived vectors, containing the replication origin of a plasmid. The basic components of a phagemid mainly include the replication origin of a plasmid, the selective marker, the intergenic region (IG region, usually contains the packing sequence and replication origin of minus and plus strands, a gene of a phage coat protein, restriction enzyme recognition sites, a promoter and a DNA segment encoding a signal peptide (Azzazy and Highsmith 2002, Qi et al. 2012). Moreover, phagemids often contain an amber stop codon, to allow host specific expression of pIII fusion protein or soluble fusion partner (Hoogenboom et al. 1991) and a gene encoding one coat protein that will be fused to the foreign DNA that is to be expressed (Barbas et al. 1991, Kang et al. 1991).

Phagemids can maintain themselves as plasmids, resulting in the expression of the desired protein in the bacteria. However, they lack other genes that encode proteins necessary for assembly of phage particle. To get production of viable phage, an infection with a helper phage is necessary. The helper phage provides the genes for the phage proteins that are missing on the phagemid. The packing signal in the helper phage genome has been altered to be less effective, and thus the recombinant ssDNA of phagemid is packaged into phage particles using helper phage proteins (Bass et al. 1990).

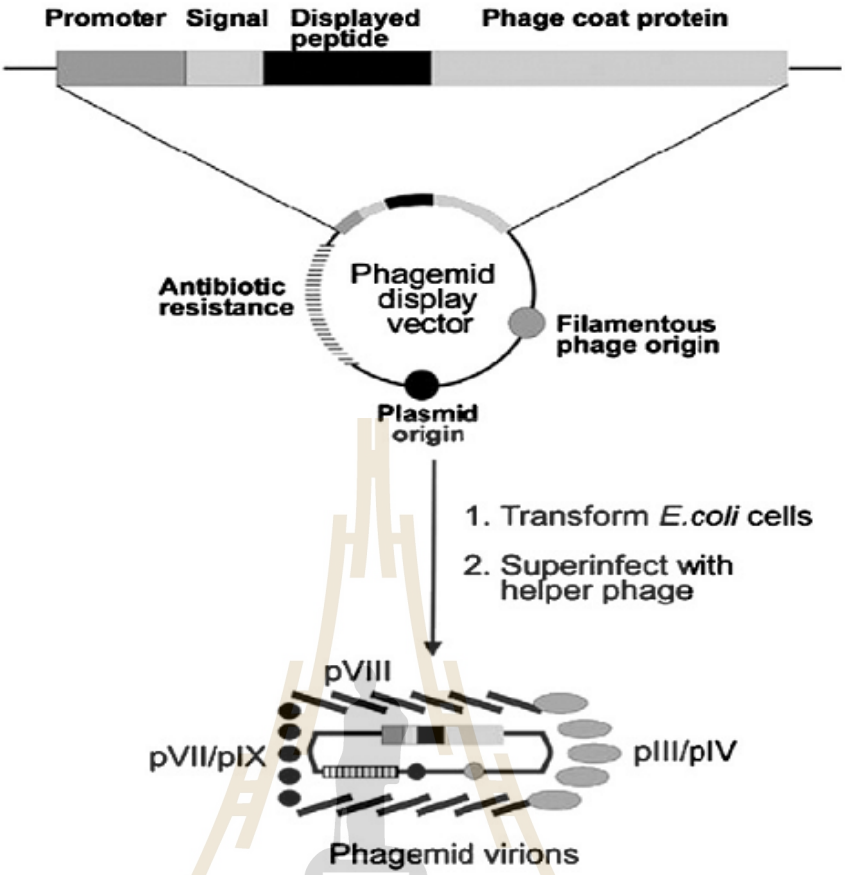

Figure 2.4 Phagemid display vector. A "typical" phagemid display vector contains origins of replication for double-stranded DNA and ssDNA synthesis (plasmid and filamentous phage origins), an antibiotic resistance gene providing selection of transformed bacteria and a fusion gene under the control of a regulated promoter (Qi et al. 2012).

There are many different types of phage-display libraries. In most cases the recombinant protein is expressed on either gpIII or gpVIII referred to as the type 3 and type 8 libraries respectively (Smith and Petrenco.P.A. 1997, Adda et al. 2002). There are three general classes of phage display systems. The first is based on the natural filamentous phage genome, the ssDNA vector. Libraries constructed by introducing foreign DNA inserts into the phage genome will result in the fusion gene

product displayed on all the coat proteins. The second system entails the use of plasmid vectors, also known as phagemids. Third, a “hybrid system,” which still utilizes the phage genome but which contains both a wide-type phage gene and a fusion gene, can be employed (Huang et al. 2012). To distinguish between these systems based on the expressed protein, Smith coined the terms “3,” “3 3,” and “33,” respectively (Smith and Petrenco.P.A. 1997). In the type 3 libraries, a gene insert encoding the recombinant protein can be included as a fusion fragment within the phage genome such that all copies of gpIII are expressed as a chimera with the foreign protein. Alternatively, if the phage genome contains genes for both the wild type and recombinant gpIII, then a mixture of recombinant and wild type gpIII molecules are produced and incorporated into the viral particles. This system is referred to as type 33.When the foreign protein is large, as in the case of antibody libraries, the gene sequence encoding recombinant gpIII is contained on a phagemid within the phage particle. Helper phage which contain a defective origin of replication, are used to produce the wild type phage proteins, such that the resulting phage particle contains the phagemid DNA and variable (ie. 0 - 5) copies of the recombinant gpIII molecule. These are referred to as the type 3+3 libraries. A series of random peptide libraries displayed on gene VIII, referred to as type 8 libraries are also available. These parallel the type 3 libraries in that they include types 8, 88 and 8+8 (Smith and Petrenco.P.A. 1997, Bratkovic 2010).

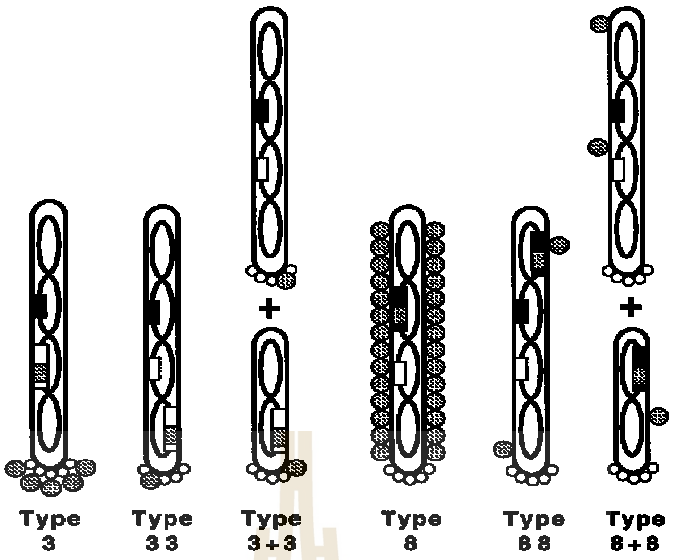

Figure 2.5 Types of phage display systems (Smith and Petrenco.P.A. 1997).

2.3.3.4 Selection of antibody libraries: "bio-panning"

Antibody libraries are screened and enriched for antigen-specific clones by a technique known as bio-panning in which phages displaying scFv are incubated with an immobilized antigen of interest (Nissim et al. 1994). A selection cycle basically contains four stages: (i) incubation of target molecules with a phage display library, (ii) washing off unbound phage, (iii) elution of the bound phage, and (iv) amplification of the eluted phage. This selection cycle is illustrated in Figure 6. Ideally, only one cycle of selection should be required, however the binding of nonspecific phage limits the enrichment that can be achieved per cycle. In practice, several rounds of selection are necessary (average 2-4 cycles). Several biopanning strategies are discussed below.

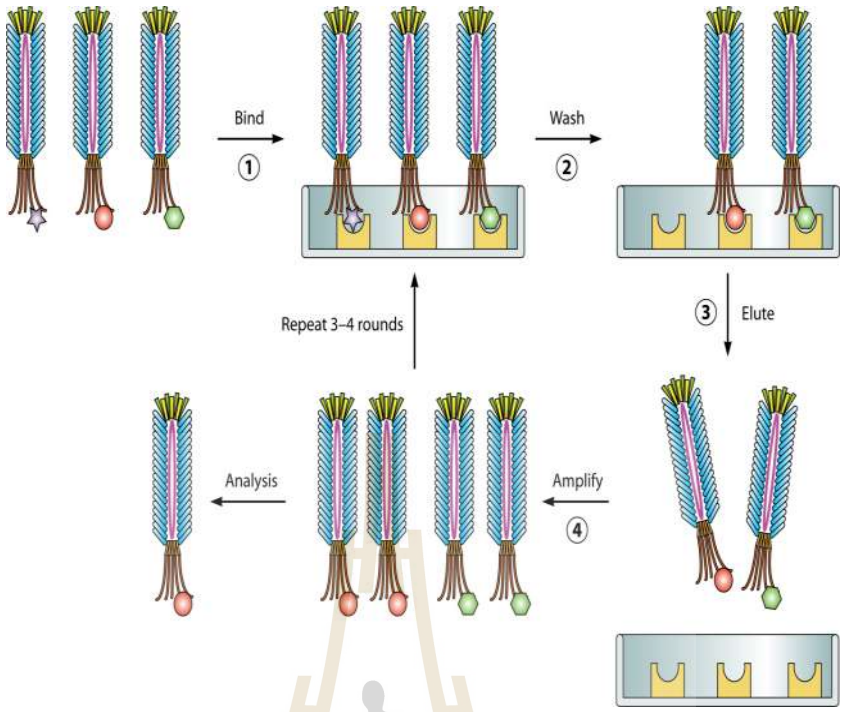

Figure 2.6 Biopanning of a phage display library to select phage binding to an immobilized target (Huang et al. 2012).

Although the principle of biopanning technique is simple, the outcome can vary due to multiple factors, such as library complexity, nature of the target, binding affinity and avidity, and other multiple experimental parameters. Even with an excellent selection strategy, the experiment will fail if the desired peptide/antibody is not present in the library. Binding affinity and avidity are other factors that need to be taken into consideration (Huang et al. 2012).

Selection using immobilized antigens: Phage libraries are selected by flowing through an affinity column with the immobilized antigen of interest (Clackson et al. 1991). Following washing of the column to remove nonspecific clones, specific binders are eluted and amplified in *E. coli*. Selection can also be performed against antigen adsorbed onto plastic surfaces such as immunotubes or enzyme-linked immunosorbent assay (ELISA) plates (Marks et al. 1991).

Alternatively, antigen may be immobilized on chips of BIAcore sensors (Malmborg et al. 1996).

It should be noted that selection of the immobilization method must take into consideration the conformational integrity of the immobilized antigen. Some phage antibodies selected against an adsorbed antigen may not be able to recognize the native form of the antigen. One way to circumvent such problem is to employ indirect antigen coating through the use of antigen-specific capture antibodies (Sanna et al. 1995).

Selection using antigens in solution: This technique allows solution binding and overcomes issues with conformational changes that are encountered upon coating antigens on solid surfaces. The use of labeled soluble antigens also allows a more accurate quantification of the antigen used during selection and consequently enhances the ability to use lower concentrations of the antigen to favor selection of high-affinity phage antibodies. Following incubation of phage-antibodies with biotinylated antigen, phage bound to the labeled antigen are recovered with avidin or streptavidin-coated paramagnetic beads. Specific phages are then dissociated from the antigen and characterized (Hawkins et al. 1992). One disadvantages of this technique is that antistreptavidin antibodies will also be isolated. However, this problem can be resolved by a depletion step using streptavidin-coated beads (Azzazy and Highsmith 2002).

Selection on whole cells: Direct selection of antibodies against markers on cell surfaces may be carried out on either monolayers of adherent cells or on cells in suspension. Unbound phage can be washed away by rinsing tissue culture flasks (monolayers) or centrifugation (cell suspension). To optimize the isolation of

antigen-specific binders and minimize the binding of irrelevant binders, a simultaneous positive and negative selection may be applied. In this approach, a competition is set up between a small number of antigen-positive target cells and an excess of antigen-negative “absorber” cells to bind antibodies of phage library; the absorber cells serve as a sink for the nonspecific adherence of irrelevant binders. A fluorescently labeled antibody against an irrelevant antigen present only on the target cells is added and FACS (Fluorescence-activated cell sorting) is used to isolate the target cells binding the specific phage antibodies (de Kruif et al. 1995). Similar approaches can be utilized to identify putative tumor-specific antigens and provide a quick high-yield approach for isolating self-replicative antibody fragments directed against novel or conformationally dependent cell surface markers. Another group subjected a scFv library to three rounds of positive selection on human melanoma cells and negative selection on human peripheral blood mononuclear cells (Kupsch et al. 1999). Selections may also be carried out on tissue sections as well as whole tissues (Azzazy and Highsmith 2002).

The advantages of whole-cell phage display are easy to see. In the case of when antigen is unavailable or the antigen is not stable under immobilization conditions, whole-cell phage display panning is normally the best choice. In addition, it is also useful in the discovery of unknown antigens. The biopanning procedure typically requires no prior knowledge of the cell surface biomarkers, allowing for the isolation of targeting peptides for cell types for which little is known about the cellular profile. For whole-cell screening, the cellular targets are identified in a two-part process. First, peptides or antibodies are first identified by screening whole cells against a phage display library. Second, the binding peptides or antibodies are tested individually in functionally based screens. In all cases, activity is confirmed in

functional assays; one does not need to either purify or identify a particular receptor in advance. Since whole cells are used as the affinity matrix, the receptors are likely to be in their native conformation, and a large variety of receptors are being screened at one time. It should be noticed that the cell surfaces would share a high degree of similarity and that the peptides would be recognizing abundant, common receptors. Thus, additional negative selections are necessary to avoid unexpected cell specificity of selected peptides (Huang et al. 2012).

The in vivo selection: In this method phage repertoires are directly injected into animals and then tissues are collected and examined for phage bound to tissue-specific endothelial cell markers as was demonstrated for peptide phage. Pasqualini et al. (1996) were the first to isolate phage-displayed peptides that home to selective vascular beds *in vivo*. *In vivo* panning has several advantages: (i) the isolated phage-displayed peptides home selectively to "intact" targets of interest; (ii) an inherent blocking step is included where most of the phage-displayed peptides that recognize ubiquitous plasma and cell surface proteins are eliminated; (iii) these peptides may be useful for the functional analysis of new receptors and potential identification of novel drug target candidates because some of the isolated peptides have been found to bind to endothelial receptors expressed in the vasculature of specific tissues.

2.3.3.5 Application of phage display

Applications for phage-displayed peptide libraries: Phage display of random peptides: Synthetic oligonucleotides with a constant length but with unspecified codons, randomized through site-directed mutagenesis using degenerate

oligodeoxynucleotides, are cloned as fusions to one of the coat proteins of M13 phage where they are expressed as peptide-capsid fusion proteins (Azzazy and Highsmith 2002). Phage-displayed peptide libraries can be used to isolate peptides that bind with high specificity and affinity to virtually any target protein. These binding peptides can be used as reagents to understand molecular recognition, as minimized mimics for receptors, or as lead molecules in drug design (Sidhu 2000).

Mapping antibody epitopes: Fragments of DNA that encode parts of the protein antigen are fused to a gene encoding one of the capsid proteins. Phage particles displaying antigenic peptides can be used for mapping epitopes of monoclonal and polyclonal antibodies (Hill et al. 1996). Phage display libraries of random peptides have also proven useful for identifying antibody epitopes in cases in which the antigen is not available or even not yet known (Cortese et al. 1994).

Phage-antibody applications: Phage-displayed recombinant antibodies have several advantages over monoclonal antibodies generated by hybridoma technology. In comparison to the time-consuming and labor-intensive cell screening processes of hybridoma production, antibody genes can be cloned directly from spleen cells using rapid recombinant DNA methods. Generation of a large natural display library from variable gene repertoires can eliminate animal immunization and large-scale cell culture for hybridoma development and allow isolation of antibodies with high affinity against any antigen. Phage display is particularly useful in cases where monoclonal antibodies could not be obtained by classical hybridoma technique such as antibodies against nonimmunogenic or toxic antigens. Phage displayed antibodies have stable genetic source. Phage antibody technology can also be used to clone and rescue monoclonal antibodies from

genetically unstable hybridomas. Phage antibody genes can be easily sequenced, mutated, and screened to improve antigen binding. Finally, soluble recombinant antibodies (not displayed on phage) can be produced quickly and economically and can be used as *in vitro* diagnostic reagents (Azzazy and Highsmith 2002).

Phage display has been used widely for identification of specific antibodies against pathogen targets. These targets are generally subdivided into two categories: (i) molecular targets, such as replication/cell division enzymes and host-pathogen virulence factors, and (ii) whole bacterial cells. In comparison with specific molecular targets, cell-based screening has the advantage in that it is an assumption-free strategy with the potential to recognize cell surface structures that may not have been considered targets using genomic-based approaches or that have not yet been identified. Using live pathogens as the target also has the advantage that all "druggable" targets on the cell surface are screened simultaneously in their native physiological context, thus allowing for the selection of potential antimicrobial activity from the outset. Antigens on the cell surface of pathogens are appealing targets for biologics because they provide potential binding sites for molecules to interfere with bacterial division (Lock et al. 2008) colonization, and virulence (Rasko et al. 2010). Both strategies have been widely applied for developing novel diagnostic tools and therapeutic treatments for infectious diseases (Huang et al. 2012).

Phage enzymes: Several enzymes have been displayed on M13 bacteriophages and retained their catalytic activities. These include alkaline phosphatase (McCafferty et al. 1991), trypsin (Corey et al. 1993), and β-lactamase (Siemers et al. 1996). In theory, any enzyme that can be expressed in *E. coli* may also be displayed on M13 phage.

Phage display libraries based on suitable enzymes can improve diagnostics by enhancing the stability and catalytic activities of enzymes, and probably enabling the engineering of catalysis that is modifiable by antigen binding.

Application of phage display technology in detection and monitoring of microorganisms: Conventional bacterial detection, diagnostic methods, especially pathogenic bacteria and virus, largely rely on microbiological and biochemical analysis which can be sensitive but overly time consuming, cost-ineffective and non-amenable to integration for on-site diagnosis.

Today, phage display is recognized as a powerful tool for selecting novel peptides and antibodies that can bind to a wide range of antigens, ranging from whole cells to proteins and lipid targets. Phage display has been used widely for identification of specific peptides and antibodies against pathogen targets. These targets are generally subdivided into two categories: (i) molecular targets, such as replication/cell division enzymes and host-pathogen virulence factors, and (ii) whole bacterial cells. Both strategies have been widely applied for developing novel diagnostic tools and therapeutic treatments for infectious diseases. (Huang et al. 2012).

Staphylococcus aureus, one of the most important human pathogens, has become a major threat to human health. Soykut et al. (2008) has used a peptide-phage display library to identify peptides binding to Staphylococcal enterotoxin B produced by *S. aureus*, is a pyrogenic toxin responsible for staphylococcal food poisoning in humans and has been an attractive choice of biological aerosol weapon due to its inherent stability and high intoxication effect.

Listeria monocytogenes is a severe food-borne pathogen that causes life-threatening listeriosis. To avoid infection by *L. monocytogenes*, it is important to detect low levels of the pathogen in food samples. Paoli and colleagues used phage display to identify a scFv antibody that can only bind to *L. monocytogenes* (Paoli et al. 2004). Later, in 2007, a surface plasmon resonance (SPR) sensor was developed based on the scFv antibody by Nanduri et al. (2007). *L. monocytogenes*-specific scFv-displayed phage was immobilized on the sensor surface to detect *L. monocytogenes* at a detection limit of $2x10^6$ CFU/ml. The some results about application of phage display technique in detection of microorganisms were summarized in Table 2.3

Table 2.3 The phage display targets of some microorganisms.

Target	Library	Potential application	Reference
Molecule target			
SEB (staphylococcal enterotoxin B)	12-mer peptide library	Anti-*S. aureus*	(Soykut et al. 2008)
SEB (staphylococcal enterotoxin B)	ScFv library	Anti-*S. aureus*	(Singh et al. 2010)
Staphylococcal enterotoxin A and B	ScFv library	Diagnosis	(Liang et al. 2011)
Surface layer protein of *Campylobacter fetus*	peptide library	Anti-*C. fetus*	(Zhao et al. 2012)
Lipopolysaccharide (LPS) of *Ralstonia solanacearum*	ScFv library	Diagnosis	(Griep et al. 1998)
Clostridium difficile toxin B	ScFv library	Diagnosis	(Deng et al. 2003)
Plasmodium vivax duffy binding protein	ScFv library	Diagnostics, vaccine design	(Kim et al. 2007)
S. aureus SdrC	12-mer peptide library	Anti-*S. aureus*	(Barbu et al. 2010)
P. aeruginosa MurA	C7C cyclic peptide, 12-mer peptide library	Anti-*P. aeruginosa*	(Molina-Lopez et al. 2006)
P. aeruginosa MurC	C7C cyclic peptide, 12-mer peptide library	Anti-*P. aeruginosa*	(El Zoeiby et al. 2003)
Esp and Intimin of *Escherichia coli* O157:H7	ScFv library	Diagnosis	(Kühne et al. 2004)
Intimin virulence of *Escherichia coli*	ScFv library	Diagnosis	(Menezes et al. 2011)
Surface epitopes of *Phytophthora infestans*	ScFv library	Diagnosis	(Gough et al. 1999)
Bhlp 29.7 protein of *Brachyspora hyodysenteriae*	ScFv library	Diagnosis	(Lobová et al. 2008)
Fumonisin B1 of *Fusarium verticillioides*	ScFv library	Detection	(Lauer et al. 2005)
H. pylori surface protein	ScFv library	Anti-*H. pylori*	(Cao et al. 2000)
Cell wall-bound proteins of *Fusarium verticillioides*	ScFv library	Detection of *Fusarium verticillioides*	(Hu et al. 2013)
Cell wall-bound proteins of *Aspergillus flavus*	ScFv library	Detection of *Aspergillus flavus*	(Xue et al. 2013)
σ^{54} factor of *Pseudomonas putida*	ScFv library	Detection	(Jurado et al. 2012)
Liposaccharide/lipid A	ScFv libraries, peptide libraries	Anti-Gram-negative bacterial agents	(Griep et al. 1998, Hayhurst et al. 2003)
Lipopolysaccharide (LPS) of *Burkholderia mallei*	ScFv libraries	Detection of *Burkholderia mallei*	(Kim et al. 2011)
SapA protein of *Campylobacter fetus*	Peptide libraries	Detection	(Zhao et al. 2012)
Protective antigen of *Bacillus anthracis*	ScFv libraries	Detection of *Bacillus anthracis*	(Wang et al. 2006)
HrpA of *Pseudomonas syringae*	ScFv library	Diagnosis	(Yang et al. 2013)
Botulinum neurotoxin A light chain	ScFv libraries	Anti-botulinum neurotoxin	(Miethe et al. 2014)

Table 2.3 (Continued).

Target	Library	Potential application	Reference
Capsular of *Burkholderia pseudomallei*	ScFv libraries	Detection of *Burkholderia pseudomallei*	(Kim et al. 2011)
Pseudonitzchia pungens toxin domoic acid	ScFv libraries	Anti-toxin domoic acid	(Finlay et al. 2006)
Crf antigen of *Aspergillus fumigatus*	ScFv library	Diagnosis	(Schütte et al. 2009)
OmpD of *Salmonella Typhimurium*	ScFv library	Diagnosis	(Meyer et al. 2011)
Shiga toxins (Stxs) of *Escherichia coli*	ScFv library	Diagnosis	(Neri et al. 2011)
Ts1 toxin from *Tityus serrulatus*	ScFv library	Anti-scorpion	(Amaro et al. 2011)
Staphylococcal enterotoxins of *Staphylococcus aureus*	ScFv library	Anti-toxin	(Chen et al. 2014)
Plasmodium falciparum histidine rich protein 2	ScFv library	Diagnosis	(Leow et al. 2014)
Toxoplasma gondii MIC2 protein	ScFv library	Against parasite antigens	(Hoe et al. 2005)
Hepatitis C virus proteins	ScFv libraries, peptide libraries	Diagnostics, vaccine design	(Bugli et al. 2001, Bugli et al. 2009)
Hepatitis A virus antibodies	9-mer peptide library	Diagnostics	(Larralde et al. 2007)
Hepatitis E virus capsid protein	Antibody library	Anti-HEV infection	(Schofield et al. 2000)
Phosphoprotein of Newcastle disease virus	ScFv libraries	Anti-virus	(Li et al. 2014)
H5N1 HA	Fab library	Anti-H5N1 infection	(Lim et al. 2008)
Transmembrane envelope glycoprotein gp46 of maedi-visna virus	ScFv libraries	Diagnosis	(Blazek et al. 2004)
p25 protein of the Maedi-Visna virus	ScFv libraries	Diagnosis	(Celer et al. 2003)
Eimeria acervulina surface antigen	ScFv libraries	Diagnostics, vaccine design	(Kim et al. 2001, Park et al. 2005)
Vesicular stomatitis virus (VSV) nucleocapsid	ScFv libraries	Diagnosis	(Cortay et al. 2006)
E protein and N protein of severe acute respiratory syndrome virus	ScFv libraries	Diagnosis	(Liu et al. 2004)
CoV spike protein of severe acute respiratory syndrome virus	ScFv libraries	Diagnosis	(Lee, Leu, Hu, et al. 2007, Lee, Leu, Hung, et al. 2007, Zhao et al. 2007)
Porcine reproductive and respiratory syndrome virus-N protein	12-mer peptide library	Detection of PRRSV	(Ren et al. 2010)
VP2 protein of infectious bursal disease virus	ScFv libraries	Diagnosis	(Xu et al. 2014)
S Protein of porcine epidemic diarrhea virus	scFv libraries	Diagnosis	(Zhu et al. 2013)

Table 2.3 (Continued).

Target	Library	Potential application	Reference
Whole cell target			
L. monocytogenes	ScFv library	Diagnostics of *L. monocytogenes* infection	(Nanduri et al. 2007)
P. aeruginosa	9-mer peptide, 12-mer peptide	Diagnostics of *P. aeruginosa* infection	(Carnazza et al. 2008)
H. pylori	ScFv library	Diagnostics of *H. pylori* infection	(Sabarth et al. 2005)
Eimeria acervulina merozoite	ScFv library	Diagnostics, vaccine design	(Zhao et al. 2010)
Neisseria meningitides group B	ScFv library	Diagnosis	(Stacy et al. 2003)
Lawsonia intracellularis	ScFv library	Diagnosis	(Dezorzová-Tomanová et al. 2007)
S. typhimurium	8-mer peptide library	Diagnostics of *S. Typhimurium*	(Sorokulova et al. 2005)
Burkholderia mallei	ScFv libraries	Detection of *Burkholderia mallei*	(Zou et al. 2007)
Ceratitis capitata	ScFv libraries	Detection of *C.capitata*	(Monzó et al. 2012)
Fusarium verticillioides	ScFv libraries	Detection	(Hu et al. 2013)
Brucella melitensis	ScFv libraries	Diagnosis	(Hayhurst et al. 2003)
Plasmodium falciparum	ScFv libraries	Diagnosis	(Wajanarogana et al. 2006)
Spores of *Bacillus*	ScFv libraries, peptide libraries	Detection of *Bacillus* species	(Zhou et al. 2002, Knurr et al. 2003, Turnbough 2003, Williams et al. 2003, Brigati et al. 2004, Mechaly et al. 2008, Walper et al. 2012)
Venezuelan equine encephalitis virus (VEEV)	ScFv library	Detection of VEEV infection	(Kirsch et al. 2008)
Chlamydia trachomatis EBs	ScFv library	Detection of *C. trachomatis*	(Lindquist et al. 2002)
Eimeria acervulina merozoite	ScFv library	Detection of *Eimeria acervulina*	(Zhao et al. 2010)
Grapevine virus B	ScFv library	Detection of virus	(Saldarelli et al. 2005)
Tomato spotted wilt virus	ScFv library	Detection of virus	(Griep et al. 2000)
Infectious haematopoietic necrosis virus	ScFv library	Detection of virus	(Liu et al. 2014)
Foot and mouth disease virus	ScFv library	Detection of virus	(ShengFeng et al. 2003)
Rabies virus	scFv library	Anti-virus	(Pruksametanan et al. 2012)
Virulent infectious bursal disease virus	scFv library	Detection of virus	(Sapats et al. 2003, Sapats et al. 2005, Sapats et al. 2006)
Severe acute respiratory syndrome virus	scFv library	Detection of virus	(Liu et al. 2005)

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All chemical reagents are molecular biology grade. Yamo1, a human non-immunized ScFv library, was constructed in laboratory using B-lymphocytes from 140 healthy people in the Northeastern Thailand (Pansri et al. 2009). *Escherichia coli* TG1 [*(lac-proAB) Sup E thi hrd* D5*/F' tra D36 pro* $A^{+}B$ *lacI*^{q} *lacZΔM15]* and HB2151 [K12 *ara (lac-proAB) thi*/F'*pro*$A^{+}B$ *lacI*^{q} *lacZΔM15*] was obtained from the MRC, Cambridge, UK, and used for cloning and amplification of phage, or production of soluble ScFv fragments, respectively. The anti-M13/HRP detection kit was purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden) and Protein L peroxidase HRP will be from Sigma. *Bradyrhizobium* sp. DOA9 was isolated from *A. americana* L. in Thailand in our laboratory (Noisangiam et al. 2012).

3.2 Instruments

These are all instruments that we used in this work.

Table 3.1 Instruments and brands.

Instrument	Brand
Autoclave	Hiclave HA-3000MIV, Hirayama, Japan
Balance	Precisa 205A, Precisa Instruments, Switzerland Precisa 3000C, Precisa Instruments, Switzerland
Centrifuge machine	Sorvall RC5C plus, Kendro laboratory Products, USA Eppendrof centrifuge 5810 R, Eppendrof, USA
Deep freezer -70 ^{O}C	Heto, Ultra Freeze, Denmark
ELISA reader	Sunrise, TECAN, Austria
Electroporator	Eppendrof 2510, Eppendrof, USA
Freezer -20 ^{O}C	Heto, HLLF 370, Denmark MyBio LFT420, DAIREI, Denmark
Gel Document set	White/Ultraviolet Transilluminator GDS7500, UVP, USA Digital Graphic Printer UP-D890, Sony, Japan
Gel dryer	Drygel sr. SLAB GEL Dryer model SE1160, Hoefer Scientific Instruments, USA
Gel electrophoresis apparatus	Mini Protean® 3 cell, BioRad, USA
Heat Box	HB1, Wealtee Corp., USA
Incubator shaker	C24 Incubator shaker, New Brunswick Scientific, USA
Incubator	Memmert, BE 500, WTB Binder BD115, Shel-Lab 2020 Low Temperature Incubator, Sheidon, USA
Laminar hood	Holten LaminAir HBB 2448, Denmark BH2000 Series ClassII Biological Safety Cabinets BHA120 & BHA180, Clyde-Apac
Membrane transfer machine	Semi Phor, Hoefer Scientific instruments, USA

Table 3.1 (Continued).

Instrument	Brand
Microcentrifuge	Mini spin plus, Eppendrof, USA Eppendorf 54154, Eppendorf, Germany
pH meter	Ultra Basic pH meter, Denver Instruments, Germany
PCR machine	DNA Engine PTC 200 peltier Thermal cycler, MJ Research, USA
Rotator	Certomat TCC, B. Braun Biotech International, Germany
Sequencing machine	ABI prism model 310 Genetic Analyzer, Applied Biosystems, USA
Shaker	Innova 2300 platform shaker, New Brunswick Scientific, UK Certomat TC2, B. Braun Biotech International, Germany
Sonicator	Waken GE100 Ultrasonic processor, Japan
Spectrophotometer	Ultrospec 2000, Pharmacia biotech, UK
Stirrer	Variomag Electronicrührer Poly 15, Germany Magnetic stirrer MSH300, USA
Thermomixer	Thermomixer compact, Eppendrof, USA
Vortex	Vortex-Genie 2 G506, Scientific Industries, USA

3.3 Methods

3.3.1 Rabbit immunization with *Bradyrhizobium* sp. DOA9

3.3.1.1 Antigen preparation

Rabbits were selected for immunization. Rabbit antiserum was developed against *Bradyrhizobium* sp. DOA9 as described previously (Somasegaran and Hoben 1994). The *Bradyrhizobium* sp. DOA9 strain was cultured in yeast mannitol medium flask in 28°C and 250 rpm for 5-7 days (Hoben et al. 1994). Cells were centrifuged at 4500 rpm at 4°C in 10 minutes and discarded supernatant. The

pellet was re-suspended in sterilized saline buffer by using a vortex mixer. The cell concentration was adjusted to approximately 1×10^{9} cells ml^{-1} by diluting with saline buffer. Then, the cell suspension was boiling for 1 h at 100°C to inactivate any remaining flagella antigens. The merthiolate was added to achieve a final concentration of 1:10,000 as a preservation. The stock solution was stored at -20°C until use (Somasegaran and Hoben 1994).

3.3.1.2 Immunization and antisera development

The healthy, young rabbit (6-12 months) was used for antiserum development. Three rabbits were used for the development of each antiserum. The rabbit was immunized by ear (intravenous) injections. The rabbit was rolled in a large towel, tightly securing the fore and rear limbs. The vein of rabbit was exposed by shaving a small section of the ear with a razor blade. Swab the shaved area with alcohol (70%) and inject the antigen with a syringe fitted with a narrow (25 gauge) needle (Somasegaran and Hoben 1994). The schedules for immunization were done following the schedule (Table 3.2).

Table 3.2 The schedule of immunization of rabbit (Somasegaran and Hoben 1994).

Day	Method	Antigen
1	intravenously (IV)	0.5 ml working solution (WS)
2	IV	0.5 ml WS
3	IV	1.0 ml WS
4	IV	1.5 ml WS
5	IV	2.0 ml WS
6-12	Resting	
13	Test bleed and perform agglutination titer	

3.3.1.3 Trial bleeding for titer determination

Blood was first bleed from intravenous (3-5 ml) to test the titer prior to get more blood by cardiac puncture. The rabbit was tied to the inclining bleeding rack. The area above the sternum was sterilized with 70% alcohol. The blood was drawn from heart with a 50-ml syringe equipped with a 26-gauge needle. Collect 20-30 ml of blood in a test tube. Then, the blood was centrifuged at 4500 rpm in 10 minutes. The supernatant was transferred into the new sterile tube and stored in -20^{0}C (Somasegaran and Hoben 1994). The titer of serum was determined by the agglutination method (Somasegaran and Hoben 1994).

3.3.1.4 Agglutination method

Firstly, the stock antiserum was diluted as follows: Arrange 10 test tubes (16×125 mm) in a row on a test-tube rack. Label them 1 through 10. Then, 9.6 ml of saline buffer was added into tube 1. For tubes 2-10, adding 2.5 ml of saline buffer. Accurately pipette 0.4 ml of the stock antiserum into tube 1. Mix the saline and serum thoroughly by sucking the serum-saline mixture into the pipette and then expelling the contents. This process will be repeated five times. Expelling should be done gently to avoid frothing. This tube now contains anti-serum of a 1/25 dilution. After that, 2.5 ml of diluted serum from tube 1 was transferred to tube 2, then mix well by using a fresh pipette (the dilution of the serum in tube 2 became 1/25×1/2 = 1/50). The dilution was repeated successively from the previous tube to the next until reaching tube 10 by using a fresh pipette tip each time.

Performing agglutinations in microtiter tray: The process was started with the highest dilution (tube 10). The 500 µl of diluted antiserum were transferred from tube into well A10 of the plastic agglutination tray by using a clean

pipette. Then, the process was repeated until all the dilutions of the antiserum were dispensed into the respective wells of row A of the agglutination tray. After that, the 500 µl of the homologous antigen (approximately 1×10^9 cells ml^{-1}) were dispensed into each of the wells from well Al through A10 by using pipette. Then, the 500µl of serum of 1/25 dilution were transferred into well A11 and then 500 µl of saline were added into this well with another calibrated Pasteur pipette. This serves as the serum-saline control. Next, the 500µl of saline were placed into well A12 and then the 500 µl of antigen were also added into this well. This serves as the antigen-saline control. All wells (Al through A12) were sealed with a strip of cellophane tape. The agglutination tray was placed in a water bath at 37°C for 2 h and then transferred to a refrigerator before reading the reactions. Figure 7 shows the steps for the antiserum titer determination in wells. The highest dilution of the serum for positive agglutinations was read and recorded. Positive agglutination appeared as granular clumps with clear supernatant. Negative agglutinations were indicated by cells settling on the bottom of the well and turbid supernatant.

To calculate the titer (serum titer is the reciprocal of the highest serum dilution at which positive agglutination occurs). The highest dilution of the serum that has positive agglutination was multiplied by two. This is because equal volumes of the diluted serum and antigen were titrated in the well. Example: If positive agglutination was detected at 1/3200 dilution of the serum, the true titer will be 3200×2 = 6400 (Figure 3.1).

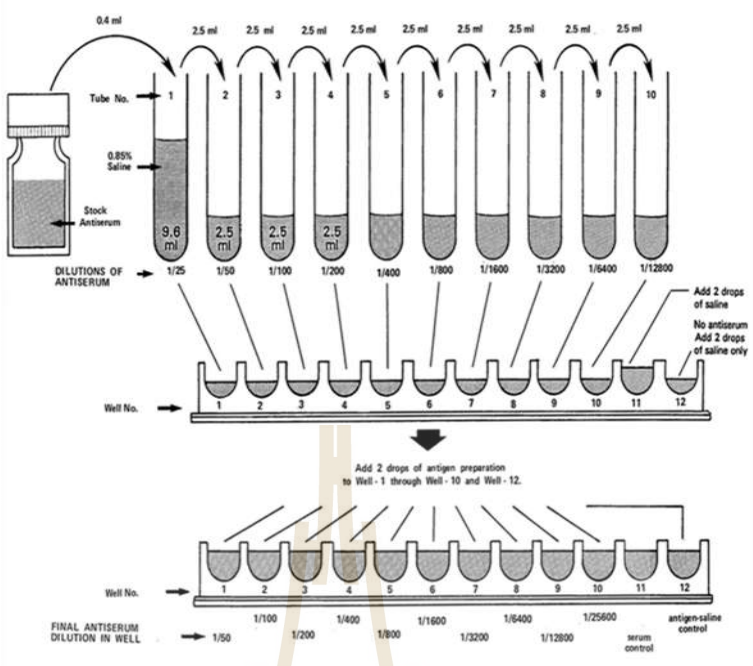

Figure 3.1 Scheme for antiserum titer determination in wells (Somasegaran and Hoben 1994).

Collecting blood and giving booster injections: Once the titer was satisfactory (not less than 1:1600), blood was transferred into a sterile screw-cap test tube of 50-ml capacity. After the blood has been clotted and refrigerated, decant the serum and centrifuge at 5000×g for 15 min to clear the serum of red blood cells. Transfer the clear serum supernatant into an appropriate container for storage by freezing. Serum was stored in in suitable-sized vials. If the titer was too low in the trial bleeding (less than 1:1600), give a booster injection of 1 ml of antigen subcutaneously immediately after the titer determination (Somasegaran and Hoben 1994). This serum was used as polyclonal antibody against *Bradyrhizobium* sp. DOA9 to compare with antibody derived from other methods.

3.3.2 Phage display library construction from immunized rabbit

3.3.2.1 Isolation of total RNA from rabbit spleen cells

Seven days after the last boost, spleen cells from immunized rabbit with strain DOA9 were prepared for mRNA extraction. Total RNA was extracted from spleen cell by TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The spleen was cut roughly up into small pieces with sterile scissors in a sterile Petri dish under liquid nitrogen, and the pieces were transferred into a sterile small mortar. Then, 1 ml of Trizol reagents were added into the mortar. The spleen tissues were homogenized with pestle under liquid nitrogen. After that, the homogenized solution was transferred to a 1.5 ml tube and 0.1 ml of chloroform was added. After shaking vigorously for 15 seconds, the homogenized solution was centrifuged at 13000 rpm at 4ºC for 15 min. Then, the clear aqueous upper layer was carefully pipetted off from the lower organic layer, and the upper layer was transferred to a fresh tube. An equal volume of isopropanol was added to the tube. The tube was incubated at room temperature for 10 minutes. The precipitated RNA was pelleted by centrifugation at 13000 rpm for 15 minutes at 4°C. The pellet was washed with 0.5 ml of 75% ethanol and then centrifuged at 13000 rpm for 15 minutes at 4°C. After the supernatant was removed, the pellet was air dried for 5 minutes at room temperature and dissolved in sterile deionized water. Then 1 µl of RnaseOut (40U/µl, Invitrogen, USA) was added into total RNA solution to remove RNase and stored at -70°C.

3.3.2.2 Synthesis of cDNA

The RNA was converted to first strand cDNA using reverse transcriptase enzyme with oligo-dT_{18} and random hexamer primers. The first strand synthesis will be started at the 3' end of poly $(A)^+$ mRNA by using the oligo-dT primer. The total volume of the reaction was 100 µl which consisted of 10 µg RNA, 2 µM oligo-dT primer, 8 ng of random hexamer primers, and 0.125 mM of dNTPs (Promega, USA), 200 units of MMuLV reverse transcriptase (200U/µl, NEB, USA) and 160 units of RNaseOut (40U/µl, Invitrogen, USA), all were dissolved in 1 × RT buffer. The RNA was heated to at 90°C for 5 minutes and quickly chilled on ice before it was added to the reaction. The reaction was incubated at 42°C for 2 hours and then the reaction was heated to 95°C for 3 minutes and quickly chilled on ice. After that, the PCR reaction was performed as described below. The second strand synthesis was done using the mRNA/DNA as a template. The cDNA was kept at -20°C.

3.3.2.3 Amplifying and reassembling heavy and light chain sequences

The genes for variable regions of heavy chain, κ light chain, and λ light chain (VH, Vκ, and Vλ) were amplified separately and recombined by three subsequent PCR reactions. The first set of PCR consists of 79 independent reactions to generate variable domains of the heavy and light chains. The heavy chain 5' primers were designed to include a *Sfi*I site, and the light chain 3' primers include a *Not*I site (Table 5). Light chain 5' primers were designed to include part of the linker region $(Gly4Ser)_3$ and compatible with the heavy chain 3' primers. The total volume of each reaction was 50 µl which consisted of 2.5-5 µl of reaction from previous step, 1 µM of each forward and reverses primers, 200 µM of dNTPs, 1× ThermoPol buffer,

2.5 units of *Taq* DNA polymerase enzyme (5U/µl, NEB, USA), 1.25 units of *Pfu* DNA polymerase enzyme (3U/µl, Promega, USA) and 0.1 mg/ml of BSA. The PCR cycles were initiated by pre-denaturing at 94°C for 5 minutes, following by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57-65°C for 1 minute, extension at 72°C for 2 minutes. The final extension was performed, by heating the reaction to 72°C for 10 minutes. The heavy chain primers were modified to include a *SfiI* site and linker sequence whereas the light chain primers were generated a *NotI* site and a linker sequence. Equal amount of PCR products were pooled into collections of VH, Vκ, and Vλ gene repertoire, and purified from the low melting temperature agarose gel according to standard protocol.

In the second PCR, heavy and light chains were assembled and amplified using *Pfu* DNA Polymerase (Promaga, USA). The assembly PCR reaction contained equal molar mixture of the pooled heavy (VH) DNA and pooled light (Vκ, or Vλ) gene repertoire. The total volume of the PCR reaction was 50 µl, which consisted of equal molar amounts of DNA, approximately 500-1,000 ng, 200 µM of dNTPs, and 1.25 units of *Pfu* DNA polymerase enzyme (3U/µl, Promega, USA), all in 1× pfu DNA polymerase buffer. The separated heavy and light chain fragments were converted to scFv by amplification under the following conditions: five cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 50 seconds and extension at 72°C for 1 minute. The correctly linked products from the assembly step were extended by PCR under the following conditions: 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 2 minutes. This was followed by the final extension at 72°C for 10 minutes.

The third reaction created a full-length scFv gene repertoire from the second PCR by PCR amplification in the presence of pull-through primers. This

PCR extended the scFv gene from the *Sfi*I and *Not*I sites flanking scFv genes, using the following primers: PTfw (5'-CCT TTC TAT GCG GCC CAG CCG GCC ATG GCC-3') and PTrv (5'-CAG TCA TTC TCG ACT TGC GGC CGC ACG-3'). The reaction was performed using the *Taq* polymerase and 1 µl of assembled products from the second PCR. This pull-through PCR was cycled 30 times (94°C for 1 min, 60°C for 1 min, 72°C for 2 min), and a final extension at 72°C for 10 min. Then the samples were purified by a QIAquick PCR Purification Kit (QIAGEN, Germany) for the next step.

3.3.2.4 Cloning of scFv fragments into pMOD1 vector

The scFv fragments DNA were inserted into pMOD1 vector (Pansri et al. 2009) between *Sfi*I and *Not*I sites. The DNA of scFv fragments and pMOD1 vector was sequential digested with *Sfi*I (20U/µl, NEB, USA) and *Not*I (10U/µl, NEB, USA) enzymes, respectively, to generate compatible sticky ends. The digestion reactions of scFv fragments and pMOD1 vector were performed separately, each in a total volume of 500 µl. For the *Sfi*I digestions, the reaction mixtures consisted of 10 µg of insert DNA, 12 µg of vector DNA, 1x NEB buffer 2, 1 µg/ml BSA and 200U of *Sfi*I (20U/µl, NEB, USA). The reactions were incubated at 50°C for 16 hours. The *Sfi*I digested DNA was cleaned by Wizard clean up kit (Promega, USA) before the next digestion step. The *Not*I digestion mixtures consisted of 400 µl of purified *Sfi*I digested DNA, 1× NEB buffer 3, 1 µg/ml BSA and 100 U of *Not*I (10U/µl, NEB, USA). The reaction mixtures were incubated at 37°C for 16 hours. After the digestion, the *SfiI*/*Not*I digested vector was dephosphorylated by adding 3 µl of CIP enzyme (10U/µl, NEB, USA) and incubated at 37ºC for 1 hour. After the dephosphorylation, the vector was inactivated by heat at 65ºC for 15 minutes. The

inserts and vectors were separated from stuffer fragments by gel electrophoresis followed by Wizard clean up kit (Promega, USA). The scFv DNA was ligated into pMOD1 vectors at a 3:1 ratio. The total volume of ligation reaction was 200 µl, which consisted of 2.8 µg of inserted DNA, 5.5 µg of pMOD1 vector, 1× T4 DNA ligase buffer and 15 µl of T4 DNA ligase (400U/µl, NEB, USA). After 16 hours of incubation at 16ºC, the ligation reaction was concentrated to 40 µl by precipitating overnight with 3 M sodium acetate pH 5.2 plus absolute ethanol. The ligation reaction was then transformed into 600 µl of *E.coli* TG1 cells by electroporation method. The reaction was done in two separate cuvettes by pipetting 20 µl of ligated sample into a 2 mm cuvette containing 300 µl of *E.coli* TG11 competent cells. The electroporation was performed at 2.5 kV, 25 µF, 200 Ω and τ approximately 4 msec using an electroporation machine (Eppendrof 2510, Eppendrof, USA). The cuvette was flushed immediately with 3 ml of SOC medium (20g Bacto Tryptone, 5g Bacto Yeast Extract, 2ml of 5M NaCl, 2.5ml of 1M KCl, 10ml of 1M $MgCl_2$, 10ml of 1M $MgSO_4$, 20ml of 1M glucose, 1L distilled H_2O) at room temperature, and the two separate transformation reactions were combined in 50 ml polypropylene tube. The 6 ml of combined transformed cells were incubated at 37ºC for 1 hour. After that the transformed cells were spread on eight 24×24 cm plates, containing TYE medium (10 g tryptone, 5 g yeast extract, 8 g NaCl and 15 g bacto-agar in 1 L distilled H_2O), 100 µg/ml ampicillin plus 1% glucose, and incubated overnight at 37ºC.

The size of the library was quantified by spreading dilutions of the transformation reaction on separate plates. A volume of 100 µl from transformation reactions was taken and a four-step 10-fold serial dilution was made. Then 100 µl of non-diluted and the four dilutions were spread on separate TYE agar plates containing 100 µg/ ml ampicillin and 1% glucose. The vector ligation control was performed in

parallel to evaluate the background of library. Ligation efficiency was also determined by counting the number of colonies from no-insert ligation.

The large plates of library were scraped into 20 ml of 2×YT medium (16 g tryptone, 10 g yeast extract and 5 g NaCl in 1 L distilled H_2O) with 20% glycerol and aliquoted into freezing vials. The glycerol stock of library was stored at -70ºC. The library size was determined by serial plating on TYE plates containing 100 µg/ ml ampicillin and 1% glucose and incubated overnight at 37°C.

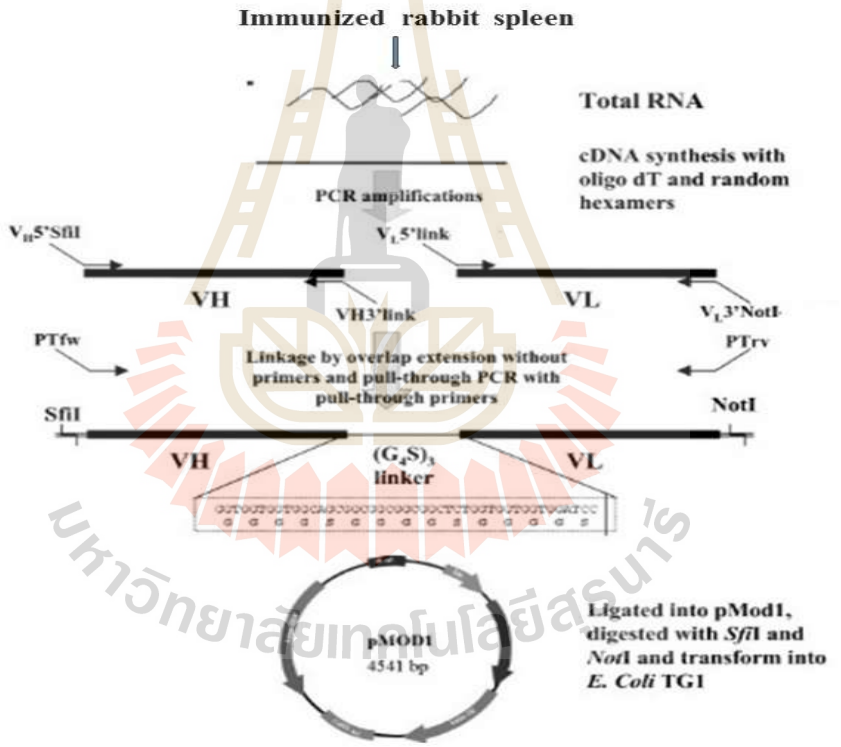

Figure 3.2 Schematic outline of the strategy used for the construction of recombinant scFv antibody library from immunized rabbit (Pansri et al. 2009).

Table 3.3 Listing of primers for PCR amplification of rabbit antibody heavy and light chain variable regions.

Primer	Numbers	Sequence
VH5'SfiI	1	5'CCT TTC TAT GCG GCC CAG CCG GCC ATG GCC CAG TCG GTG GAG GAG TCC RGG 3'
	2	5'CCT TTC TAT GCG GCC CAG CCG GCC ATG GCC CAG TCG GTG AAG GAG TCC AGA 3'
	3	5'CCT TTC TAT GCG GCC CAG CCG GCC ATG GCC CAG TCG YTG GAG GAG TCC GGG 3'
	4	5'CCT TTC TAT GCG GCC CAG CCG GCC ATG GCC CAG SAG CAG CTG RTG GAG TCC GG 3'
	5	5'CCT TTC TAT GCG GCC CAG CCG GCC ATG GCC CAG TCG CTG GAG GAG TCC GGG GGT 3'
VH3'linker	1	5'GCC AGA ACC GCC TCC CCC CAT CCC TCC GCC ACC CGA TGG GCC CTT GGT GGA GGC TGA RGA GAY GGT GAC CAG GGT GCC 3'
	2	5' GCC AGA ACC GCC TCC CCC ACT CCC TCC GCC ACC GAC TGA YGG AGC CTT AGG TTG C 3'
VLκ5'linker	1	5' AGT GGG GGA GGC TCT GGC GGA GGT GGG TCG GAG CTC GTG MTG ACC CAG ACT CCA 3'
	2	5' AGT GGG GGA GGC TCT GGC GGA GGT GGG TCG GAG CTC GAT MTG ACC CAG ACT CCA 3'
	3	5' AGT GGG GGA GGC TCT GGC GGA GGT GGG TCG GAG CTC GTG ATG ACC CAG ACT GAA 3'
	4	5' AGT GGG GGA GGC TCT GGC GGA GGT GGG TCG GCT CAA GTG CTG ACC CAG AC 3'
	5	5' AGT GGG GGA GGC TCT GGC GGA GGT GGG TCG GMC MYY GWK MTG ACC CAG ACT CC 3'
VLλ5'linker	1	5' AGT GGG GGA GGC GGT TCT GGC GGA GGT GGG TCG GAG CTC GTG CTG ACT CAG TCG CCC TC 3'
	2	5' AGT GGG GGA GGC GGT TCT GGC GGA GGT GGG TCG CAG CCT GTG CTG ACT CAG TCG 3'
VLκ5'NotI	1	5' CAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC ATT GGT GCC 3'
	2	5' CAG TCA TTC TCG ACT TGC GGC CGC ACG TAG GAT CTC CAG CTC GGT GCC 3'
	3	5' CAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAC SAC CAC CTC GGT GCC3'
VLλ5'NotI	1	5' CAG TCA TTC TCG ACT TGC GGC CGC GCC TGT GAC GGT CAG CTG GGT CCC 3'
	2	5' CAG TCA TTC TCG ACT TGC GGC CGC ACC TGT GAC GGTCAG CTG GGT CC 3'

Note: S=G/C; R=G/A; K=G/T; M=A/C; Y=C/T; W=A/T; H=A/C/T; B=C/G/T; V=A/C/G; D=A/G/T; N=A/T/G/C. Bold fonts indicate linker sequence. Recognition sites for restriction enzymes (*Sfi*I/*Not*I), and linker sequence are italicized.

The quality of library was determined by restriction fragment analysis. The colonies were randomly picked into 5 ml media containing appropriate antibiotic. After the cultures were incubated for 16-18 hours at 37°C with shaking, the cultures were centrifuged at 6800xg for 3 minutes to collect the cells for DNA extraction. Then plasmid minipreparations were made from each clone (QIAgen spin Mini-prepkit, USA). Digestion reactions of DNA were performed in a total volume of 10 µl. The reaction mixtures consisted of 2 µl of DNA, 0.2 µg of vector DNA 1× NEB buffer 3, 1 µg/ml BSA, 1 U of *Nco*I and 1 U of *Not*I. The reaction was incubated at 37°C overnight and was run on 1 % (w/v) agarose gel (Emresco, USA) in TAE buffer at 100 volts for 45 minutes. The diversity of library was performed by restriction fragment analysis using *Bst*NI enzyme. The digestion reactions of DNA were performed in a total volume of 10 µl. The reaction mixtures consisted of 2 µl of DNA, 0.2 µg of vector DNA 1×NEB buffer 3, 1 µg/ml BSA, 1 U of *Bst*NI. The reactions were incubated at 65°C for overnight and were run on 1 % (w/v) agarose gel in TAE buffer at 100 volts for 45 minutes.

3.3.3 Amplification of phage library for bio-panning

To rescue phagemid library, the 500 µl library stock was add into 50 ml pre-warmed 2×YT containing 100 µg/ml ampicillin (Emresco, USA) and 1% (w/v) glucose (Carlo erba, Italy) and grow with shaking at 37°C until the cell reached to the mid-log phase (OD_{600} about 0.4). The 2×10^{11} KM13 helper phage was added into 50 ml cultures and was then incubated at 37°C without shaking for 30 minutes. The culture was centrifuged at 4,000 rpm for 10 minutes following by resuspension of the cell pellet in 100 ml of 2×YT containing 100 µl/ml ampicillin, 50 µg/ml kanamycin

(Fluka, Switzerland) and 0.1 % glucose. After that the culture was incubated with shaking at 30°C overnight. The next day, the overnight culture was centrifuged at 4,000 rpm for 30 minutes. The supernatant was precipitated by using PEG (Fluka, Switzerland) /NaCl (Emresco, USA) (20 % Polyethylele glycol 6000, 2.5 M NaCl) for 1 h on ice followed by centrifugation at 4,000 rpm for 30 minutes. All supernatant was removed; the pellet was resuspended in phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl (Emresco, USA), 8 mM Na_2HPO_4 (Merck, Germany), 1.5 mM KH_2PO_4 (AnalaR, England), pH 7.4). The phage solution was centrifuged at 10,000 rpm for 10 min. To remove any pellet, the phage library solution was transferred to new tube and stored at 4°C before biopanning. For long term storage, phage supernatant was kept in 20% glycerol (Emresco, USA)/PBS and stored at -70°C. To determine the phage titer, the PEG precipitate phage was diluted by making six 100-fold serial dilutions and adding 100 µl of diluted phage into 900 µl of mid-log *E. coli* TG1. The infected *E. coli* TG1 cells were incubated at 37°C for 30 minutes followed by plating on separate 2×YT agar containing 100 µg/ml ampicillin and 1% (w/v) glucose. The plates were incubated at 37°C overnight.

3.3.4 Biopanning against *Bradyrhizobium* sp. DOA9

The phage antibody library was used for selecting the phage particles that specifically bind to immobilized antigen. The biopanning method was done to select phage clone that specific binding with strain DOA9 from naïve human library and immunized rabbit library from the step 3.3.2 according to the protocol described by Pansri et al. (2009).

Broth culture antigen preparation for biopanning: The DOA9 strain was grown in 50 ml flask containing yeast extract mannitol broth (YMB) at 28°C, 200 rpm

for 5 days. Cell pellets were harvested aseptically in sterile saline buffer (0.85% NaCl), then washed three times in sterile saline by centrifugation at 4500 rpm, 20 min at 4°C. Cell pellets were re-suspended in saline buffer and were adjusted to 1×10^9 cells/ml. This could be estimated by reading the optical density of the suspension on a spectrophotometer. An optical density of 0.45 (OD_{600}) is approximately equal to 1×10^9 cells/ml. Then, the suspension was treated by boiling in water bath for 1 h to inactivate flagella and other protein antigens. The total protein was determined by Bradford assay (Bradford 1976) The merthiolate as a preservation was added to achieve a final concentration of 1:10,000. The stock solution was stored at -20°C until use.

The selection was done on immunotube (Nunc, Denmark) with immobilized antigen. The 20 µg antigen was immobilized in 400 µl of 100 mM $NaHCO_3$ pH 8.5. The immobilizing was performed overnight at 4ºC. Next day, the tube was washed three times with PBS. The immunotube was then blocked to avoid non-specific binding of phage particles with 2% (w/v) skimmed milk (2% MPBS) and incubated at room temperature for 2 hours with rotating. The blocking solution was poured off and the well was washed three times with PBS. Then 300 µl of 2% MPBS and containing 10^{12} phages from the phage antibody library was added to the tube and incubated at room temperature for 1 h with rotating and 1 h on bench at room temperature. The unbound phages were removed by washing three times with PBS containing 0.1% (v/v) tween 20 (PBST) and two times with PBS. After shaking out the wash buffer and repeating this washing step for ten times, the well was rinsed with PBS ten times. The bound phages could be eluted by trypsinization or/and low pH condition using acidic elution buffer (50 mM glycine-HCl pH, 2.0). The trypsinization was performed by adding 50 µl of freshly prepared trypsin buffer (5 µl of 10mg/ml

trypsin stock in 45 µl of PBS) to the well and leaving it for 10 minutes at room temperature. The low pH elution was performed by using 50 µl of 50 mM glycine-HCl pH, 2.0, to be elution buffer. After incubation at room temperature for 10 minutes, the acidic solution had to be neutralized by adding 50 µl of neutralization solution (200 mM $NaHPO_4$ pH 7.5). The recovered phages were amplified in *E.coli* TG1 cells by infecting 175 µl of mid-log phase *E.coli* TG1 at OD_{600} of 0.4 with 25 µl of eluted phages and incubating at 37ºC for 30 minutes. For the output titering, the eluted phages were diluted. Three 10-fold serial dilutions were performed. Then 100 µl of non-diluted and the three dilutions were separately spread on TYE agar plates containing 100 µg/ ml ampicillin and 1% (w/v) glucose. The plates were incubated at 37ºC overnight.

To continue with the next round of selection, 1 ml of 2 × YT media was added on agar plates containing a lawn of infected bacteria, and the cells were loosened with a glass spreader. The scraped cells were kept as a 15% (v/v) glycerol stock at -70°C, and 10 µl of scraped bacteria were added into 10 ml of 2 × YT supplemented with 100 µg/ml ampicillin and 1% (w/v) glucose, and incubated at 37°C with shaking until the OD_{600} was 0.4 (approximately 2 h). After this procedure, phage was rescued by super-infecting the cells with 5×10^{10} helper phage KM13 and incubated at 37 °C, without shaking, for 30 minutes. Afterward, the culture media was exchanged by centrifugation at 4,000 rpm at 4°C for 15 minutes, the supernatant was removed, and the pelleted bacteria were resuspended in 5 ml of 2 × YT containing 100µg/ml ampicillin, 50 µg/ml kanamycin, and 0.1% (w/v) glucose. Later, it was incubated at 30°C with shaking overnight. On the following day, the overnight culture was centrifuged at 4,000 rpm and 4°C for 15 minutes. Phage was precipitated by adding 1 ml of PEG/NaCl (20% (v/v) polyethylene glycol 6000 in 2.5 M NaCl) into 4

ml of the supernatant and kept on ice for 1 h, and later centrifuged at 4,000 rpm, at 4°C for 30 minutes. The supernatant was removed and the pellet was resuspended in 100 µl PBS for the next round of selection.

3.3.5 Individual Phage Rescue

Individual phage-infected colonies were randomly picked from the TYE plate and grown in wells of a 96-well plate (Nunc, Denmark) containing 100 µl 2 × YT plus 100 µg/ml ampicillin and 1% (w/v) glucose. After overnight incubation at 37 °C, small inocula (5 µl) from each well were transferred to a second 96-well plate containing 200 µl of 2 × YT plus 100 µg/ml ampicillin and 1% (w/v) glucose. The first plate was kept as master stock by adding glycerol to a final concentration of 20% (v/v) and kept at -20 °C. The second plate was incubated with shaking at 37 °C for 2 h, and later phage was rescued by adding 1010 helper phage to each well. Following this they were then incubated at 37 °C for 1 h before centrifugation of the plate at 4,000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 200 µl of 2 × YT containing 100 µg/ml ampicillin and 50 µg/ml kanamycin, and cultured at 30°C overnight (20 h) with shaking (250 rpm). The overnight culture was spun at 4,000 rpm for 10 minutes, and 50 µl of the supernatant-containing phage was used in monoclonal phage ELISA.

3.3.6 Screening for specific binder by monoclonal phage ELISA

Broth culture antigen was prepared with the same method for biopanning. Then, the broth culture antigen was dilute in sodium carbonate buffer and calculated with 5µg protein total per wells of 96 MicroWell™ plates (Nunc, Denmark). For negative control, wells were coated with 2% (w/v) skim milk in sodium carbonate

buffer. After incubation at 4 °C overnight, the plates were washed three times with PBS. The wells were then blocked with 4% (w/v) MPBS for 2 h at room temperature. The wells were re-washed three times with PBS. Then, one hundreds µl of phage supernatant and 50 µl of 4% (w/v) MPBS were added to each well and incubated at room temperature for 2 h. Unbound phage was removed by washing three times with PBST and three times with PBS. Subsequently, 100 µl of HRP-labeled anti-M13 (1:5000 dilution in 2% (w/v) MPBS) was added into each well. After incubation for an additional 1 h at room temperature, the wells were washed again, as described previously, and 100µl of ABTS substrate solutions were added into each well and incubated at 37°C for 30 min. The resulting absorbance was read at 405 nm by plate ELISA reader (Sunrise, TECAN, Austria).

3.4 Detection of DOA9 strain by phage ELISA

3.4.1 In pure culture sample

To determine the optimum value of antigen for phage ELSIA, the antigen was prepared as described above. Then, the broth culture antigen was diluted in sodium carbonate buffer and calculated with variable amount of total protein (4, 5, 6, and 7 µg) per wells of 96 MicroWell™ plates (Nunc, Denmark). The procedure ELISA was done according to the protocol described by Pansri, Jaruseranee et al. (2009). The broth culture antigen was dilute in sodium carbonate buffer and calculated with 5 µg protein total per wells. The wells were sealed with tape to avoid evaporation and incubated overnight at 4°C. Next day, the wells were rinsed three times with PBS and blocked with 2% skim milk in phosphate buffer saline (MPBS) for 2 hours, at room temperature with rotating. Then the wells were rinsed three times with phosphate buffer saline (PBS). After that, phage stock (about 10^{12}pfu) was added

to wells containing 50 µl of 4% MPBS. Then, wells were washed three times with PBST (PBS with 0.1% Tween 20) followed by three times of PBS. Secondary antibody was used for detecting the bound phages. The 1:5000 dilution of a mouse anti-M13 phage-horseradish peroxidase (HRP) conjugate (Amersham-Pharmacia Biotech, Sweden) in 50 µl of 2% MPBS was added into each well as secondary antibodies. The plates were incubated at room temperature for 1 h with rotating. The wells were washed as described above. The 100 µl of ABTS (2, 2-azino-di-3-ethyl-benzthiazoine-6-sulfonate) peroxidase substrate (Fluka, USA) containing 0.05% H_2O_2 was added, and the plates were incubated at room temperature for 1 h. The assay was performed in triplicate. The reaction was stopped by adding 50 µl of 1% sodium dodecyl sulfate. Detection was done by measuring the absorbance at OD 405 nm in an ELISA plate reader (Sunrise, TECAN, Austria).

To test cross reactivity of positive phage clones and polyclonal antibody, the phage ELISA was done the same with above phage ELISA with optimum value of antigen. For negative control, other bacterial strains (SUTN9-2, SUTN1-12, USDA110, SUT 47, SUT 19, TAL173 and PRC 008) and *Bacillus* sp. were used in phage ELISA. Besides, 3C1 phage clone (against aflatoxin) was used as negative phage control in the test. For polyclonal antibody, the antibody prepared from rabbit immunized with DOA9 was diluted 1:7500 in PBS buffer and add 100 µl/well containing 50 µl of 4% MPBS. The binding was done at room temperature for 1 h with rotating. Secondary antibody was used for detecting the bound phages and polyclonal antibody. A 1:5000 dilution in PBS of protein A was added for polyclonal antibody wells as secondary antibodies. The next steps was the same as above procedure.

3.4.2 In nodule sample

Nodule antigen preparation: The seeds of siratro (*Macroptilium atropurpureum*) were sterilized by soaking in concentrated sulfuric acid for 10 minutes. After that, all seeds were washed with sterilized water five times and then soaked in sterilized water overnight at room temperature under dark condition. All seeds were germinated on sterilized 0.8% (w/v) water agar for 2 days in the dark conditions at room temperature. The germinated seeds were planted in Leonard's jars and inoculated with 1 ml of 1×10^9 cells/ml of each *Bradyrhizobium* strain, DOA9, SUTN 9-2, SUTN 1-12, and USDA110. All plants were supplemented with N-free medium (Hoagland et al. 1950) and grown in a room for 1 month. After 4 weeks, nodules were collected from plants. Then, nodules were washed with sterile distilled water and stored over silica gel at room temperature. Prior to analyses, nodules were revived by distilled water for 1-2 hours (Payakapong et al. 2003).

ELISA was done according to the protocol described by Pansri et al. (2009) with some small modifications for nodule samples. Nodules were crushed and gently grinded in small mortar. Nodules were calculated with 4 nodules/wells. Then, sodium carbonate buffer (pH 8.5) was added to mortar. Finally, 200 µl of the nodule suspension was added to the wells of ELISA plate (Nunc, Denmark). The broth culture antigen was diluted in sodium carbonate buffer and calculated with 5 µg protein total per wells as positive control. The wells were sealed with tape to avoid evaporation and incubated overnight at 4°C. Then, the procedure was the same phage ELISA for pure culture sample.

3.5 Detection of DOA9 by immunofluorescence assay

Immunofluorescence assay was performed according to the protocol described by Somasegaran and Hoben (1994) with some modifications for scFv antibody against target in both sample pure culture and nodule. Negative control was made using only diluents, no phage, no polyclonal antibody and non-related phage clone. The phage clone 3C1 also was used the same phage ELISA for control.

For pure culture sample, the culture spots on the slide were incubated with 10 µl of polyclonal antibody (1:7500), phage clone (10^{12}pfu) in one hour. The slide was rinsed two times with PBS and incubated with 10 µl of secondary FITC labelled anti-rabbit Ig antibody (for polyclonal antibody) M13-FITC (for phage clone antibody). Then, slides were incubated in the moist chamber for one hour at room temperature. Unbound proteins were removed by rinsing and washing with PBS and mounted in 40% glycerol under a cover slip. The slip was examined under a fluorescence microscope (Nikon, Japan).

For nodule sample, the section (30µl) of nodule was placed on slides. Then, sections were incubated in PBS containing calcofluor white M2R (Sigma, Germany) to a final concentration of 0.01% (w/v) for staining of the plant cell wall. After washing with PBS, the procedure was done the same for pure culture samples. Finally, the slip was examined under a fluorescence microscope (Nikon, Japan).

3.6 Sequence analysis and structure modeling

Plasmid DNA from positive scFv phage clones were extracted and purified from overnight culture by using a commercially plasmid preparation kit (Mini Preps: Qiagen, Germany) and were completely sequenced by Macrogen (Seoul, Korea)

using primers: For VH use link seq new CGA CCC GCC ACC GCC GCT G and For V use pHEN seq CTA TGC GGC CCC ATT CA. Sequences of scFvs were aligned and analyzed with rabbit germline sequence via http://www.imgt.org (IMGT®, the international ImMunoGeneTics information system®). For 3D structure modeling, the nucleotide sequence was translated to an amino acid sequence using the Translate Program (Expert Protein Analysis System, Expasy).

Docking between recombinant scFv antibodies and antigen was performed to determine possible binding regions by *in silico* prediction tools of the three dimensional structure (Kelley 2009) and used the PDB file on PyMol (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC).

3.7 Production of soluble scFv antibodies

3.7.1 Subcloning and expression in *E. coli* HSM174

The genes of positive scFv antibody clones, RD6/2 and RB8 were subcloned into pET27b vectors between *Nco*I and *Not*I sites. Plasmids were purified from each clone (QIAgen spin Mini-prepkit, USA) and transformed into *E. coli* HSM 174. The *E. coli* harboring recombinant plasmid was grown at 37°C in 5 ml of M9ZB media (1% Tryptone, 0.5% Sodium Chloride, 1XM9 Salts, 0.4% Glucose, 1mM Magnesium Sulfate) containing 50 µg/ml kanamycin and 2% w/v glucose. The culture was incubated overnight with shaking at 30°C. One percent (v/v) of each overnight culture was used to inoculate into M9ZB media supplemented with 2% (w/v) glucose and 50 µg/ml kanamycin. Then, the culture was incubated with shaking at 30°C for 4 hours. After that, the cells were centrifuged at 16°C, 5000 rpm for 15 min followed by resuspension with M9ZB media containing 1% (w/v) glycerol, 50 µg/ml kanamycin and 1 mM isopropylthio-galactoside (IPTG, Emresco, USA). After continuing

incubation at 16°C for 20 hours with shaking, the cell pellet was collected by centrifugation at 16°C, 8000 rpm for 15 min for 15 min. The secreted antibody could be found in the supernatant. The hexahistidine tag was used for detection and purification.

3.7.2 Purification of soluble scFv antibody

The recombinant scFv antibodies containing a hexahistidine tag was purified using Ni++ ions immobilized on resin by covalent linkage to nitrilotriacetic acid (NTA). The supernatant was immediately applied to a Ni–NTA agarose affinity column containing 1-2 ml of bed volume (QIAGEN GmbH, Hilden, Germany), and the chromatography was carried out gravitationally at 4°C, following the Qiagen's protocol. The column was washed two times with 50 ml of wash buffer (20 mM Tris–HCl buffer, pH 8.0 and 150 mM NaCl) containing 20 mM imidazole. Ni–NTA bound enzyme was eluted with 250 mM imidazole in the same buffer. The eluted fractions were then dialyzed by snake skin dialysis bag followed by centrifugal dialysis using Amicon® Ultra-4 Centrifugal Filter Devices (Mr 10,000 cut-off, Millipore, Ireland) to remove imidazole and concentrated soluble scFv antibody. SDS-PAGE electrophoresis was used to monitor antibody purity.

3.7.3 Soluble scFv antibody ELISA

The Immuno 96 microWell™ plate (Nunc, Denmark) was immobilized with pure culture and nodule sample. The procedure is the similar with phage ELISA with some small modifications for soluble scFv antibodies. The soluble scFv antibodies were detected with His prob-HRP (1:5000). The color of the reaction was developed with ABTS reagent (Fluka, Switzerland). The reaction was quantified by measuring the absorbance at OD 405 nm.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Phage display library construction from immunized rabbit

4.1.1 RNA extraction and cDNA synthesis

The total RNA was prepared from rabbit spleen samples by TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA extracts were high purity, free of DNA contamination and allowed production of first strand cDNA by using MMuLV reverse transcriptase (NEB, USA) and a mix of oligo-dT_{18} and random hexamers primers (Figure 4.1).

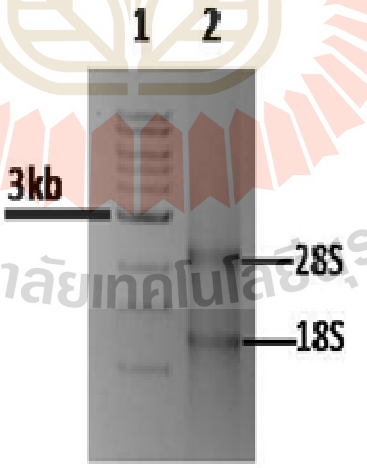

Figure 4.1 The total RNA on 1% agarose gel and stained with 0.5 µg/ml of ethidium bromide. Lane 1: 1 kb DNA marker; Lane 2: sample.

4.1.2 Amplifying and reassembling heavy and light chain sequences

In order to reduce amplification biases, 26 independent PCRs were performed using all possible combinations within a designed primer set (Table 4) that encompasses the entire theoretical set of whole repertoires of rabbit antibody genes. The PCR reactions included five VH forward primers (VH5'*Sfi*I) paired with two VH reverse primers (VH3'link) which generated a total of ten reactions; whereas three Vκ forward primers (VL5'link-κ) paired with two Vκ reverse primers (VL3'*Not*I-κ) generated a total of six reactions; and five Vλ forward primers (VL5'link-λ) paired with two Vλ reverse primers (VL3'*Not*I-λ) generated a total of ten reactions. The PCRs led to the representation in the repertoire of variable regions derived from all conceivable framework assemblies.

The scFv fragments were constructed by assembly of VH and VL genes together with linker, which generated VH-linker-VL orientation. In order to create scFv fragment genes of a VH-linker-VL type, the separate VH and VL genes were converted to a scFv gene by inserting a linker DNA sequence. The assembly of VH, VL, and linker fragment was carried out by PCR. The 3′ ends of VH gene complementary to 5′ ends of linker sequence and the 3′ ends of this linker DNA was complementary to 5′ ends of VL gene. The final pull-through PCR was done with two primers (PTfw & PTrv) compatible to the 5' *Sfi*I or 3'*Not*I segments of the heavy and light chain gene repertoires. The size of scfv fragment was approximately 800 bp (Figure 4.2).

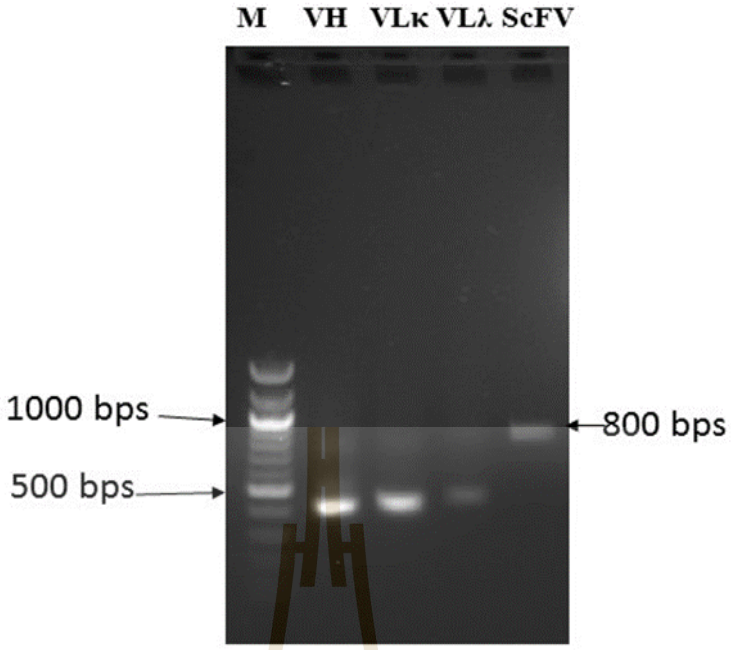

Figure 4.2 PCR products of VH and VL genes and scFv fragment on 1% agarose gel and stained with 0.5 µg/ml of ethidium bromide. M: Marker 100bp.

4.1.3 Cloning of scFv fragments into pMOD1 vectors

The fragments were digested with *Not*I/*Sfi*I and ligated into *Not*I/*Sfi*I-linearized pMOD vector. The recombinant vector was introduced into competent *E. coli* TG1 cells and the transformants were pooled. Finally, the library was obtained with 3.5×10^6 independent clones. The investigation of full-length inserts from some randomly clones using *Nco*I and *Not*I restriction enzyme digestion showed that 85% of all clones had inserted fragment (Figure 4.3).

4.1.4 Diversity analysis of antibody fragments

To analyze the diversity of the scFv repertoire and the quality of the primary library, DNA segments encoding the scFv genes from each positive clone above were examined by *Bst*NI digestion, and their fingerprint patterns were compared. The result showed that clones have different patterns (Figure 4.4).

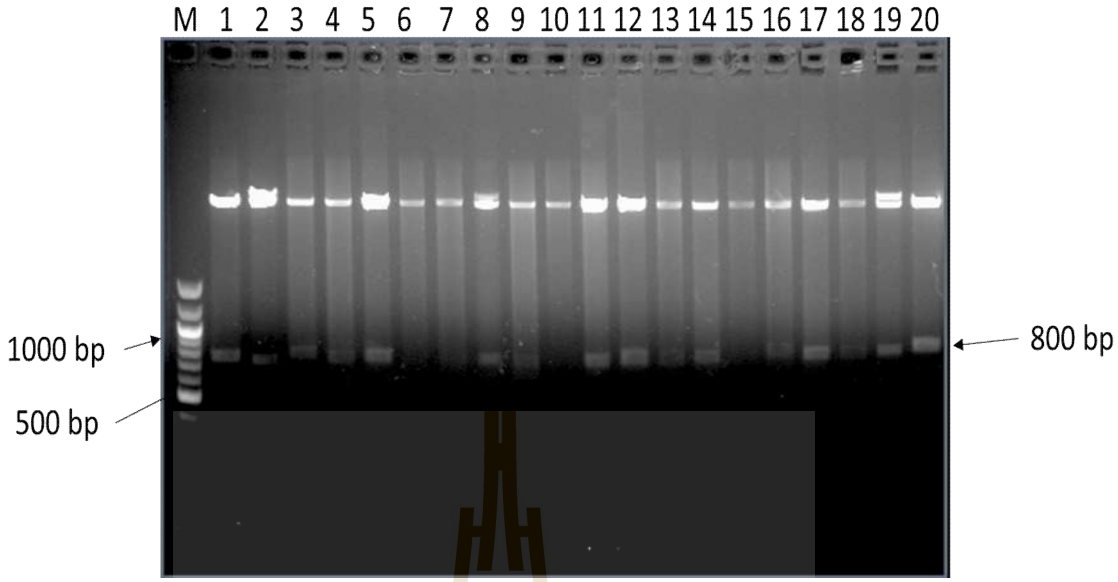

Figure 4.3 DNA fragment of random clones from unselected library after digestion with *Nco*I and *Not*I restriction enzyme on 1% agarose gel and stained with 0.5 µg/ml of ethidium bromide. M: Marker 1kb; 1-20 randomly clones.

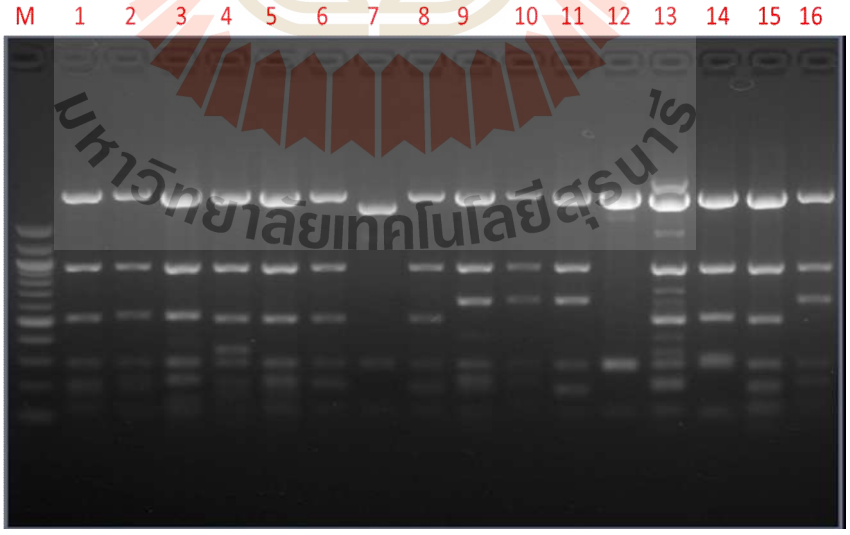

Figure 4.4 DNA fragment of random clones from unselected library after digestion with *BstN*I restriction enzyme on 1% agarose gel and stained with 0.5 µg/ml of ethidium bromide. M: Marker 100 bp; 1-16 phage clones.

4.2 Selection of scFv fragments specific to DOA9 by biopanning

In order to select the best recombinant scFv phage antibody against target, two scFv antibody libraries, naïve human library (Yamo library) and immunized rabbit library constructed in this study were used in biopanning with target antigen, DOA9 in immunotube. Three rounds of selection and amplification of the bound phages were performed. As show in Table 4.1, a significant enrichment of specific scFv phage antibody was observed during the three time panning.

Table 4.1 Selective enrichment of scFv antibodies during biopanning process with rabbit library.

Rounds	Amount of antigen (µg)	Input (CFU)	Ouoput (CFU)	Output rate %
1st	20	1.0×10^{12}	2.57×10^{4}	2.57×10^{-6}
2nd	15	1.0×10^{12}	1.72×10^{5}	1.72×10^{-5}
3rd	10	1.0×10^{12}	1.02×10^{6}	1.02×10^{-4}

Output rate (%) = (numbers of phage output × 100) / (numbers of phage input)

After each round of panning, 192 clones (two 96-well plates) were picked randomly and tested for affinity to target by phage ELISA. Finally, in third round of panning, two positive phage clones, RB8 and RG9 were selected from immunized rabbit library with high affinity with DOA9 target (Figure 4.5). Two clones, RB8 and RG 9 were used for the further studies.

Similarly, for naïve human library, only one round was performed against target DOA9. The result showed in Figure 4.6, one phage clone RD6/2 had high signal ELISA with DOA9 target. This clone was used for the further studies.

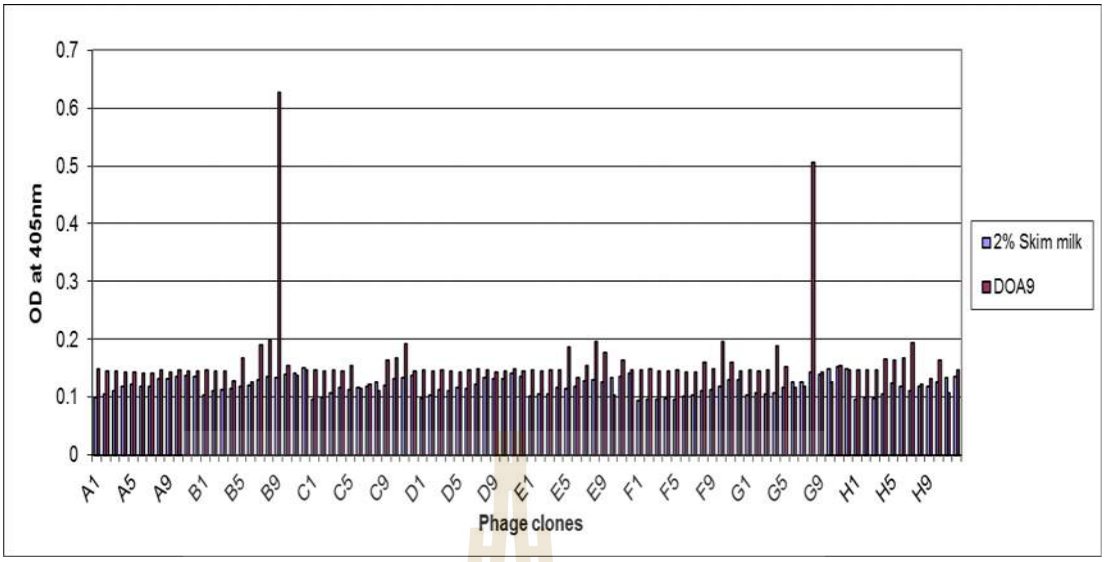

Figure 4.5 Binding of specific phage to DOA9 target from third round of panning with immunized rabbit scFv antibody library.

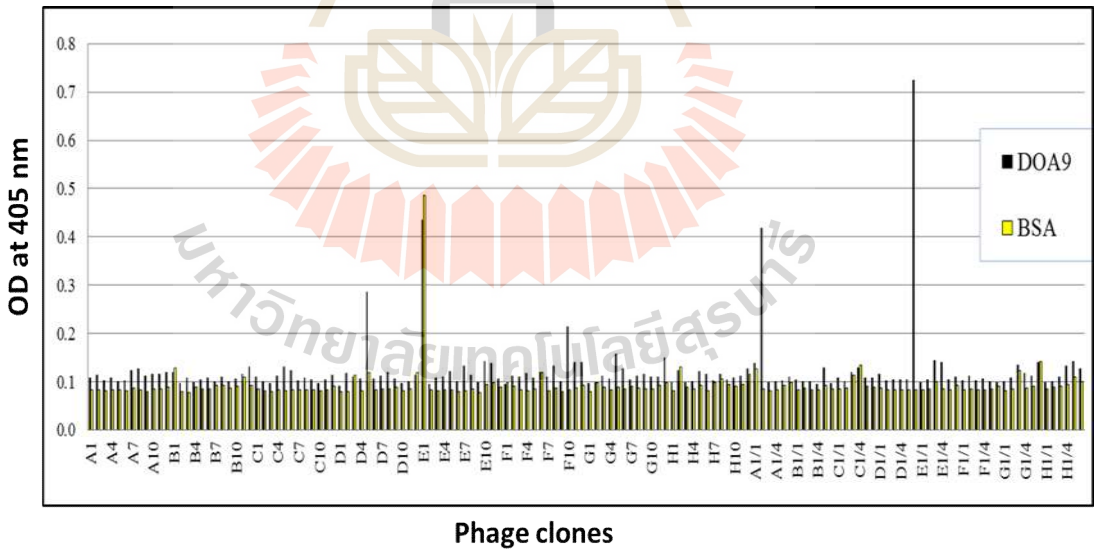

Figure 4.6 Binding of specific phage to DOA9 target from first round of panning with naïve human scFv antibody library.

4.3 Detection of DOA9 strain by phage ELISA

In order to evaluate the potential application of recombinant scfv antibody for detecting and monitoring DOA9 in production of bio-fertilizer, positive phage scfv antibody was tested with both bacterial sample in broth and in nodule by two methods, phage ELISA and immunofluorescence assay.

The limitation of antigen amount for detection by phage ELISA is 4 µg of total protein, while the optimum amount of antigen for detection was 5 µg (Figure 4.7). Then, the all ELISAs in next steps were conducted with 5 µg of protein for pure culture sample.

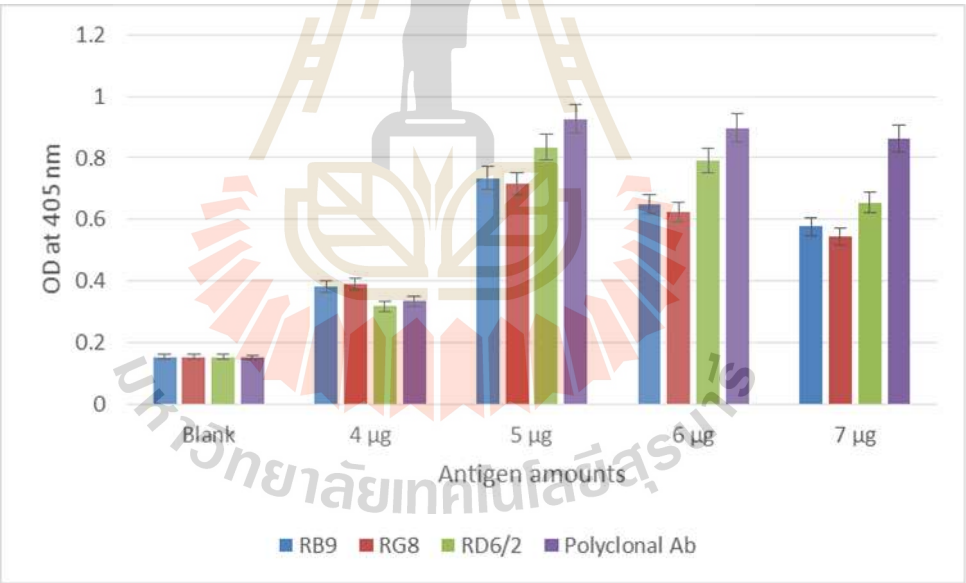

Figure 4.7 Phage ELISA of scFv clones against DOA9 in pure culture with variable antigen amounts. Value indicated mean of triplicate reactions. Error bars showed the standard derivation for each set of data. RB9 and RG8 phage clones from rabbit library; RD6/2 phage clone from human library.

The binding of three positive clones RB8, RG9, RD6/2 and poly clonal antibody were tested by phage ELISA to bind with pure culture and nodule sample were shown in Figure 4.8 and 4.9, respectively. The results showed that, phage clones had high affinity with the target DOA9 in both pure culture and nodule antigen, while polyclonal antibody still had cross reactivity with other bacterial strains. No signal was observed with negative control phage, 3C1 (specific phage clone for mycotoxin antigen). Interestingly, the phage clones have no cross-reactivity with other antigens from related rhizobial strains, USDA110, SUTN 9-2 and SUTN 1-12. These results suggest that phage clones are more specific with DOA9 than polyclonal antibody.

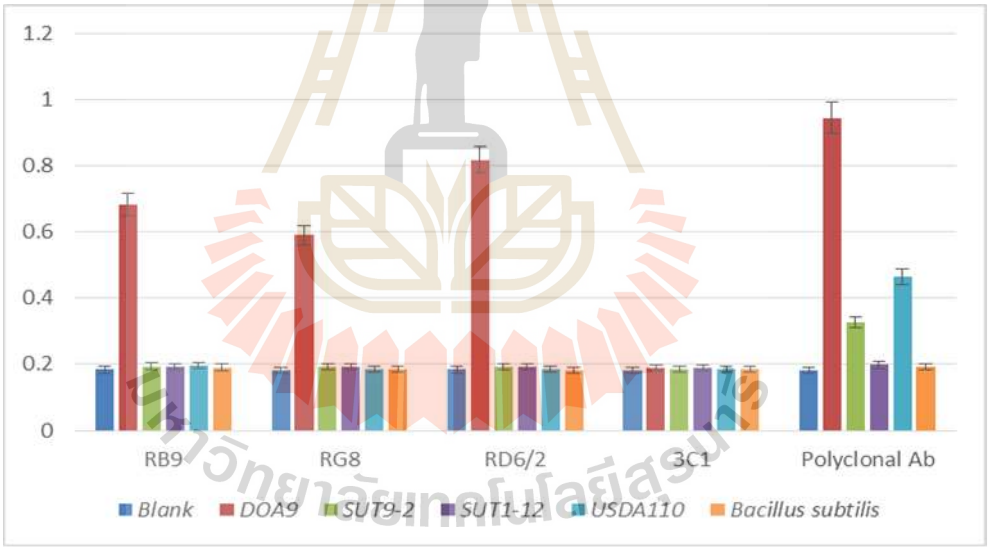

Figure 4.8 Phage ELISA of scFv clones against DOA9 and other antigens in pure culture. Value is mean of triplicate reactions. Error bars showed the standard derivation for each set of data. RB9 and RG8 phage clones from rabbit library; RD6/2 phage clone from human library; 3C1 is negative control phage clone.

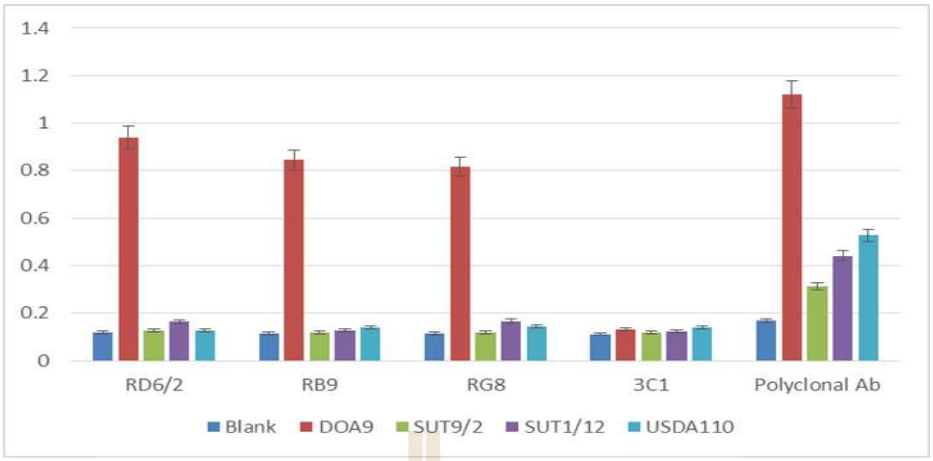

Figure 4.9 Phage ELISA of scFv clones against DOA9 and other antigens in nodule sample. Value indicated mean of triplicate reaction. Error bars showed the standard deviation for each set of data. RB9 and RG8 phage clones from rabbit library; RD6/2 phage clone from human library; 3C1 is negative control phage clone.

The phage clones were also checked with some other strains, SUT 47 (*Bacillus* sp.), SUT 19 (*Pseudomonas* sp.), TAL 173 (*Bradyrhizobium* sp.) and PRC 008 *(Bradyrhizobium* sp.). The result showed that phage clones were more specific with target DOA9 and no cross reactivity with other targets, while polyclonal antibody tended to have higher cross reactivity with tested strains (Figure 4.10).

In conclusion, the positive phage clones, RD6/2, RB8 and RG9 were specific binding to target DOA9 as well as polyclonal antibody. Phage clones also had low cross reactivity with other bacterial strains.

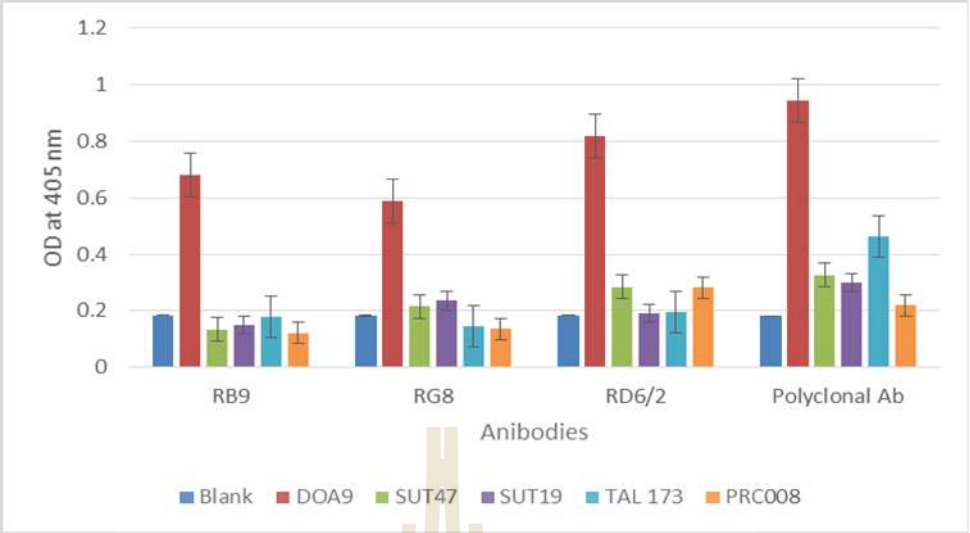

Figure 4.10 Phage ELISA for checking cross reactivity of phage clones and polyclonal antibody with other strains of commercial bacterial biofertilizer. Value indicated mean of triplicate reactions. Error bars showed the standard derivation for each set of data. RB9 and RG8 phage clones from rabbit library; RD6/2 phage clone from human library.

4.4 Detection of DOA9 strain by Immunofluorescence assay

The reactivity of phage clones RB8 and RG9 (rabbit clones) and RD6/2 (human clones) against target DOA9 were also confirmed by immunofluorescence assays and compared with reactivity of polyclonal antibody and negative phage control (3C1). The results were summarized in Figures 4.11 and 4.12. The results were the same with phage ELISA experiment.

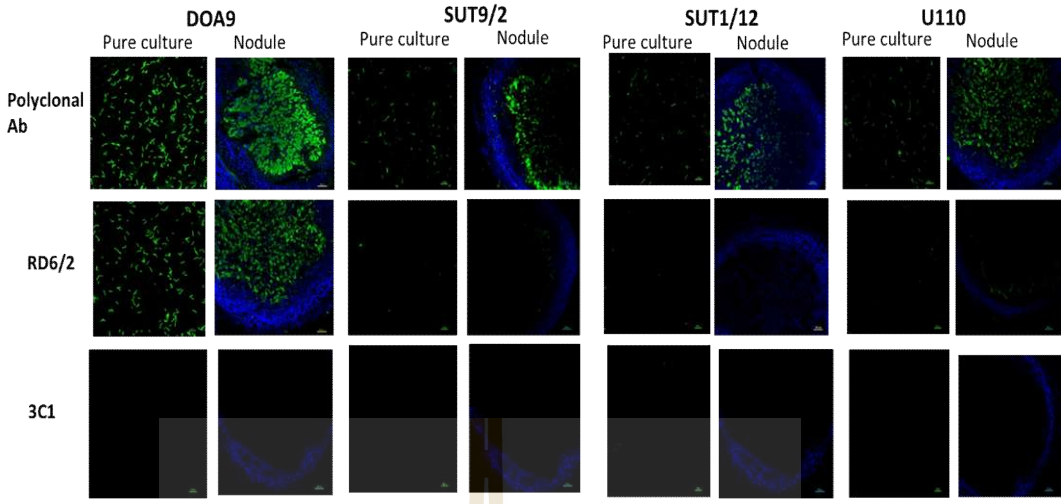

Figure 4.11 Immunofluorescence microscopy of polyclonal antibody and recombinant human scFv phage antibodies with some bradyrhizobial antigens in form of pure culture and nodule bacteroid. RD6/2 clone from naïve human library; 3C1 clone from naïve human library against aflatoxin (for negative control). For pure culture, scale bar is 10 µm and magnification is 80×; for nodule sample scale bar is 100 µm and magnification is 10×.

The selected phage clones had specific binding activity with DOA9 target antigen in both pure culture and nodule sample. No fluorescence was obtained with the negative controls which consisted of no phage, no polyclonal antibody, and mycotoxin-specific phage clone (3C1 clone). The blue color was detected as calcofluor binding with plant cell. Polyclonal antibody also still had cross reactivity with other *Bradyrhizobium* strains, such as SUT 9-2, SUT1-12, and USDA110 in both antigen of pure culture and nodule.

Visualization of DOA9 bacteroids in nodule sample and bacterial cells in pure culture sample by using fluorescence microscopic technique validated the specificity and affinity of phage antibody RB8, RG9, and RD6/2 phage clones with target DOA9.

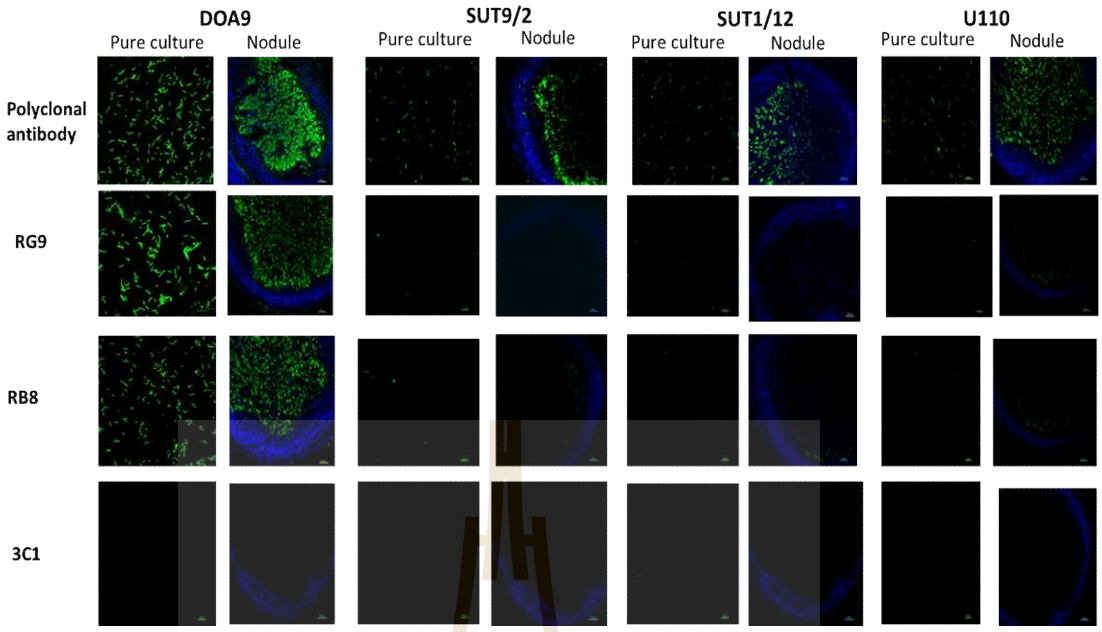

Figure 4.12 Immunofluorescence microscopy of polyclonal antibody and recombinant rabbit scFv phage antibodies with some bradyrhizobial antigens in form of pure culture and nodule bacteroid. RB8 and RG9 clones from immunized rabbit library; 3C1 clone from naïve human library against aflatoxin (for negative control). For pure culture, scale bar is 10 µm and magnification is 80×; for nodule sample scale bar is 100 µm and magnification is 10×.

4.5 Sequence analysis of specific clones and 3D structure prediction

The DNA sequence of positive clones, RD6/2, RB8 and RG9 were confirmed by automated DNA sequencing. The result indicated that the sequence of clone RB8 and RG9 were identical. Then, the DNA sequences of phage clone RB8 and RD6/2 were analyzed by using IMGT® software (http://www.imgt.org/IMGT_vquest/vquest). The result showed that the RB8 phage clone belonged to rabbit immunoglobulin VH1 heavy chain and VL λ light chain families. The RD6/2 phage clone belonged to human immunoglobulin VH4 heavy chain and VLκ light chain

families. Table 4.2 showed the origin of germline and family of the VH and VL genes and the amino acid changing with germline of the two recombinant scFv antibodies.

Table 4.2 Germlines and Families of VH and VL segments of two positive clones.

Clones	Fragment	Germline	Amino â change number	Family
RB8 (Immunized rabbit library)	VH	IGHV1S69*01	7	VH1
	VL	IGLV4S4*01	7	VLλ
RD6/2 (Naïve human library)	VH	IGHV4-59*01	17	VH4
	VL	IGKV3-20*01	0	VLκ

Figure 4.13 Deduced amino acid sequence of RB8 and RD6/2 clones, the sequence of complementary determining regions (CDR) of VH and VL chains and linker were underlined.

Figure 4.13 showed the amino acid sequence alignment of two recombinant scFv clones, RD6/2 and RB8, with the complementary determining regions (CDRs) of the VH and VL and the linker sequence domains were indicated.

Antibodies or immunoglobulins are fascinating molecules from both a functional and structural point of view. They have the ability to recognize virtually any foreign molecule with exquisite specificity and very high affinity. These properties are brought about by their three dimensional (3D) structure architecture, which consists of a tetramer of two identical pairs of polypeptide chains: the heavy and light chains. Each chain includes homologous domains that have a similar tertiary structure to each other: the so-called immunoglobulin fold. The two N-terminal domains of the polypeptide chains are called variable domains (VL and VH, respectively), and they are responsible for antigen binding. Predicting the structure of an antibody from its amino acid sequence has several important applications. The structural models obtained once such predictions have been made can be used in docking simulations to identify the region of the antigen recognized by the antibody. Predicting the structure of antibodies also has implications for the re-design of biotechnologically useful antibodies adopted in a variety of experimental setups.

For prediction of 3D structure, the nucleotide sequence of RB8 and RD6/2 were translated to an amino acid sequence using Expasy (http://web.expasy.org/translate/). The recombinant antibody structures were predicted using the Phyre program via the site at http://www.sbg.bio.ic.ac.uk/phyre2. The schematic three dimensional structure recombinant scFv antibody RB8 with CDRs was shown in Figure 4.14 and 4.15.

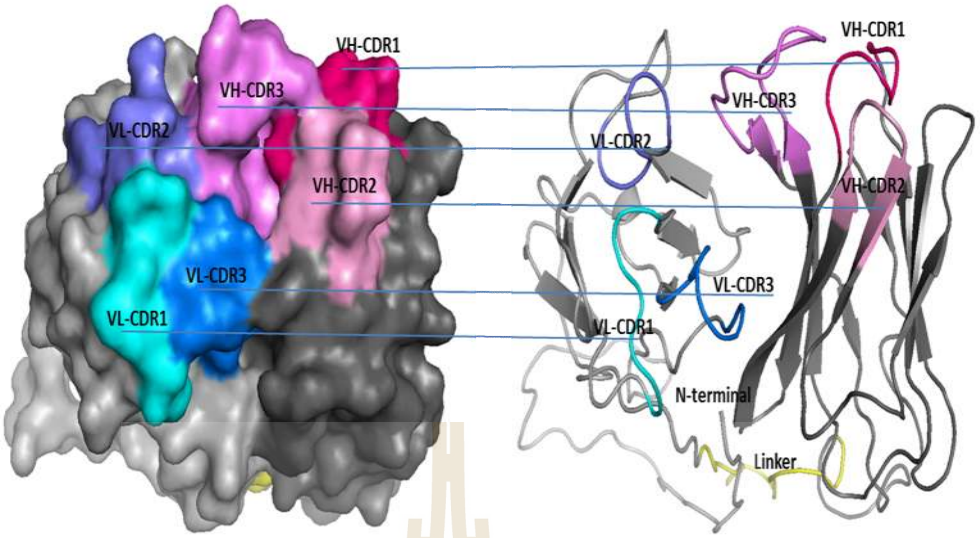

Figure 4.14 Three-dimensional structure modelling of recombinant rabbit scFv antibody RB8.

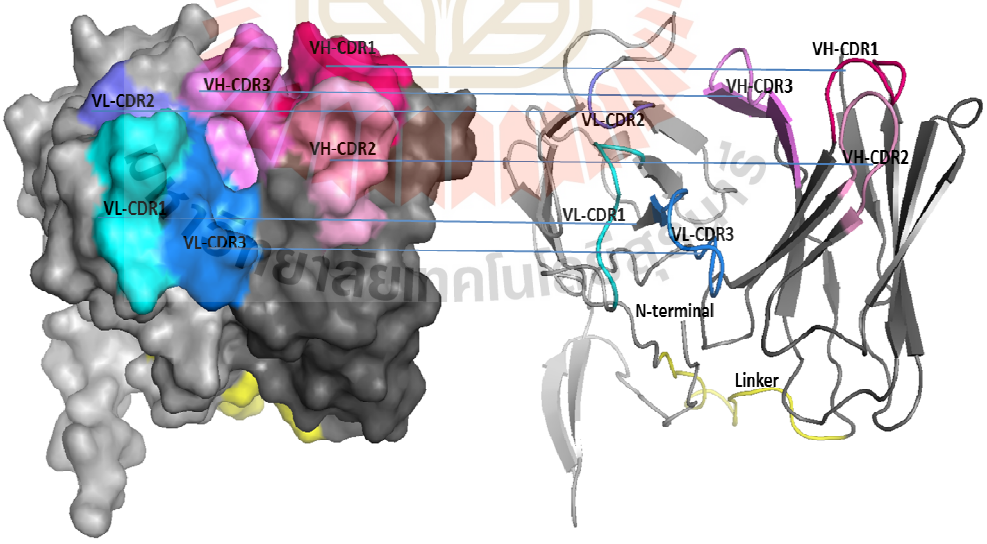

Figure 4.15 Three-dimensional structure modelling of recombinant human scFv antibody RD6/2.

4.6 Production of soluble antibody fragments

In order to express the scFv fragment in *E. coli*, the scFv genes of RD6/2 and RB8 phage clones were inserted into pET21b vector. Recombinant expression plasmid was transformed into *E. coli* HSM 174 for protein expression. This expression vector allows the expression of 6xHistidine-tag-fusion-proteins which using for purification and detection. The expression was performed overnight at 16°C with shaking. The secreted antibody could be found in the supernatant. The recombinant antibody scFv proteins were purified from the supernatant. After purification by immobilized metal ion affinity chromatography, the purify fraction were examined on SDS-PAGE gel (Figure 4.16). The results of SDS-PAGE showed that the scFv-hexahistidine fusion antibodies with a size of about 30kDa were successfully purified from the supernatant using Ni-NTA resin.

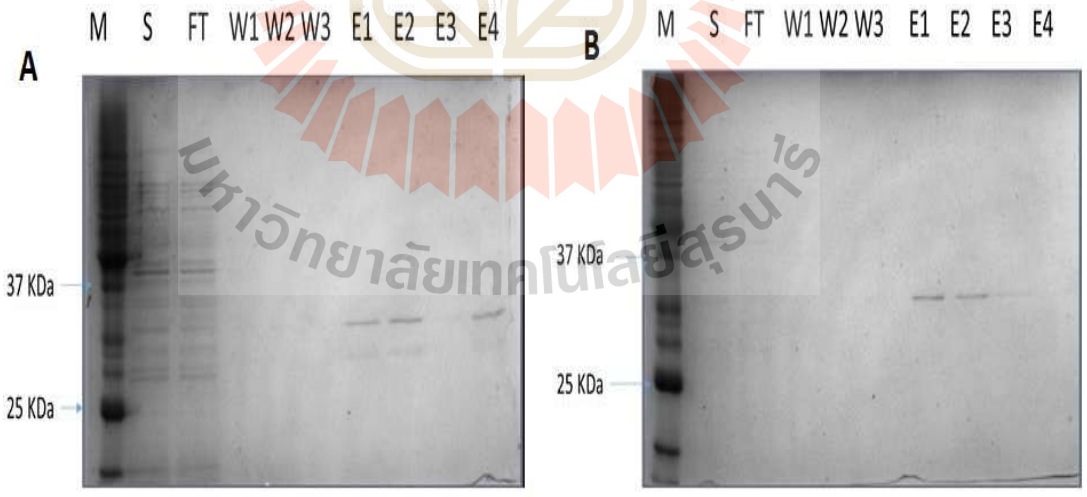

Figure 4.16 SDS PAGE of purified scFv antibody from supernatant. A: RD6/2 phage clone; B: RB8 phage clone. Lane M, protein molecular weight marker; lane S, culture supernatant fraction; lane FT, flow-through fraction; lane W1, W2, W3, wash fraction; lane E1, E2, E3, E4: Elution.

The reactivity of recombinant antibodies after purification against DOA9 was tested by ELISA. The results were shown in Figure 4.17 and 4.18. The results demonstrated that the recombinant antibodies retained a specific binding activity for target DOA9 in both pure culture and nodules form of antigen as same as the phage scFv antibodies.

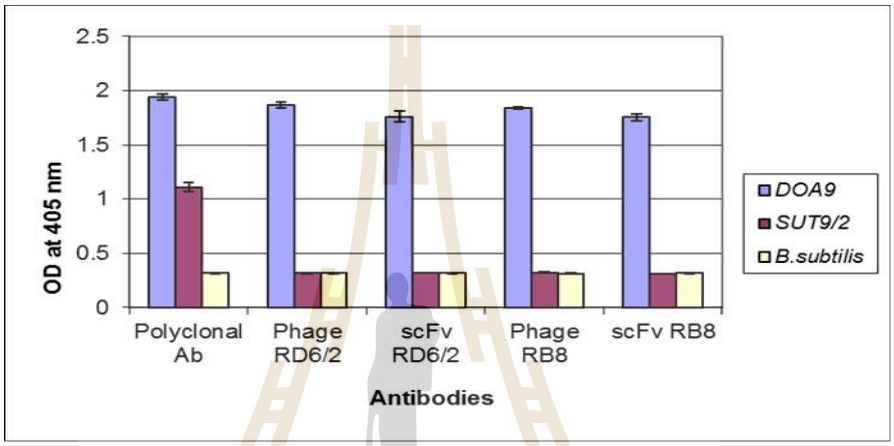

Figure 4.17 ELISA of recombinant scFv antbodies against DOA9 and other antigens in pure culture sample. Value indicated mean of triplicate reactions. Error bars showed the standard deviation for each set of data.

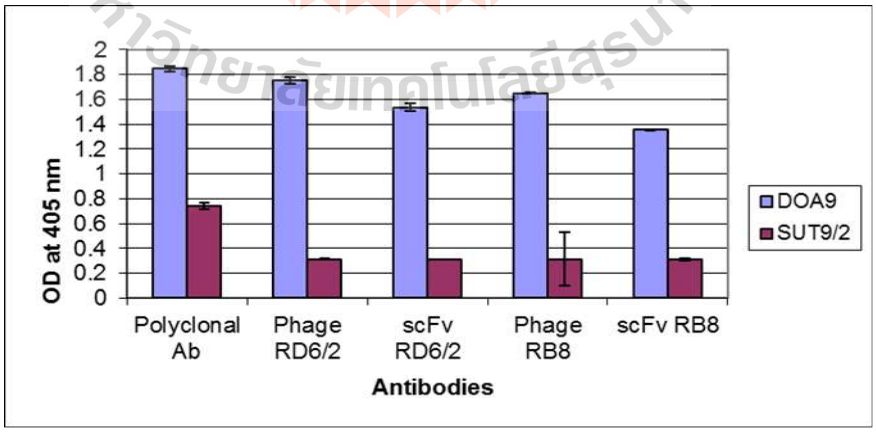

Figure 4.18 ELISA of recombinant scFv antbodies against DOA9 and other antigens in nodule samples. Value indicated mean of triplicate reactions. Error bars showed the standard deviation for each set.

4.7 Discussions

Since DOA9 may have potential to be used as biofertilizer, DOA9 was used as a model in this study for development a new method for detection and monitoring of DOA9 in both pure culture and in form of bacteroid in legume nodules. Up to now, many techniques useful in the analysis of rhizobial inoculants have been developed by using antisera. However, it is very often to have cross-react with other rhizobial strains within the same species. This cross-reactivity may also extend to other rhizobial biovars or species and sometimes even to members of other bacterial genera (Hoben et al. 1994). These cross-reactions are the main problem of using polyclonal antibody to detect and monitor specific rhizobium. In this study, the results clearly showed that polyclonal antibody produced from rabbit immunized with DOA9 have higher cross reactivity with other bacterial strains than that of phage antibodies. It may be due to these tested bacteria contain similar epitopes or antigens on their cells. Thus, polyclonal antibody which normally produced from various epitopes presented in DOA9, may have antibodies that probably bind to those similar antigens presence in other bacteria.

Therefore, monoclonal antibodies are the ideal method and precise tools for bacterial identification that provides higher reliability for detection of rhizobial cell during production and application of biofertilizer. However, production of monoclonal antibodies by hybridoma technology is high cost and time consuming method. Phage display was first reported by George P. Smith (Smith 1985). It is an effective tool for producing a large diversity of peptides and proteins, and from these selecting molecules that have specific binding properties. This technique can be particularly useful for studying protein-ligand interactions, antigen-antibody

interactions and to improve the affinity of proteins for their binding properties (Smith and Petrenco.P.A. 1997). Production of recombinant antibodies by phage display technology has many advantages, such as small amounts of antigen, large scale and low cost. Single-chain antibody fragment (scFv) is a small engineered antibody, in which the variable heavy chain (VH) and light chain (VL) of the antibody molecule are connected by a short, flexible polypeptide linker. Using scFv for detection of antigen has several advantages. First, it retains the specific affinity to the antigen, though usually lower than its original antibody; second, scFv can be produced in large quantity in bacterial expression system at low cost; and third, it is easy to be manipulated to adapt different applications, for example, fusion with protein drug to target and kill pathogens or with marker molecules for detection purposes (Huang et al. 2012). Nowadays, scFv antibodies have considered as one of potential in immunological techniques for detecting and monitoring many targets such as: bacteria, virus, toxin, pesticides and other residues in both basic research and application research.

In this study, I used two recombinant scFv antibody libraries for screening the best scFv antibodies for target, DOA9. The first library is naïve human library, the YAMO-I library. This library was constructed from 140 non-immunized (naïve) donors (Pansri et al. 2009). This library was applied in successful selections of specific antibodies against antigens, such as aflatoxin, crude snake venom, cancer cell surface, and rabies virus (Pansri et al. 2009, Rangnoi et al. 2011, Pruksametanan et al. 2012). The second library was the immunized rabbit library that constructed by phage display technology. After bio-panning, one phage clone, RD6/2 (from human library) and two phage clones, RB8 and RG9 (from rabbit library) were selected to against target DOA9 with similar affinity. Interestingly, the scFv antibody specific to DOA9

was also successfully obtained from naïve human library in this study. It could be possible that some of naïve donors may have experience exposed to some bacteria that have similar epitopes to DOA9. Moreover, it was noticed that only two scFv clones specific to DOA9 were obtained from rabbit library that immunized directly with this strain. However, it is unclear to explain this phenomenon. The recombinant scFv antibody were applied for detection of target DOA9 in both pure culture and nodule sample by phage ELISA and immunofluorescence assay comparing with polyclonal antibody against other bacterial strains. Strain SUTN9-2 and SUTN1-12 were in genus *Bradyrhizobium* isolated from *Aeschynomene americana* same as DOA9, while strain SUTN1-12 showed high similarity with strain DOA9 when compared by using gene multi locus analysis (Noisangiam et al., 2012). However, the derived phage clones could be used to distinguish or have higher specificity to DOA9 than SUTN1-12 or SUTN9-2. Moreover, other bradyrhizobia and bacteria used as commercial biofertilizer, such as *Bradyrhizobium diazoefficiens* USDA110 for soybean, *Bradyrhizobium* sp. PRC008 for mungbean, *Bradyrhizobium* sp. TAL173 for peanut, and other Plant Growth Promoting Rhizobacteria (PGPR) were included to test the cross-reactivity by using these antibodies. Since these strains have been used as biofertilizer in the field for many years and some of them may persist in the soil, it is necessary to test whether the scFv antibodies derived from this study could bind or have the cross-reactivity with these commercial biofertilizer strains. The results showed that, phage clones were more specific with target than polyclonal antibody and were also able to detect the bacteria in the root nodule. However, phage clones were not specific when applied directly for detecting the target bacterium in the soil. Thus, the result encourage more study to increasing the specific binding of the recombinant scFv antibody when applied directly with soil sample.

CHAPTER V

CONCLUSION

In conclusion, I successfully constructed the phage displayed rabbit scFv antibody library from the immunized rabbit with high quality. Then, the immunized rabbit scFv antibody library and the naïve human scFv antibody library were affinity selected against the target, *Bradyrhizobium* sp. strain DOA9 by biopanning. Two recombinant scFv antibodies were obtained from the immunized rabbit scFv antibody library. After sequencing, they were identical. From the naïve human scFv antibody library, one recombinant scFv antibody was obtained. The result was confirmed in two methods, phage enzyme linked immunosorbent assay and immunofluorescence assay with two sample types, pure culture and bacteroid in plant nodule. These antibodies have higher specificity than that of the polyclonal antibody and no cross-reactivity with another tested bacterial strains. This study described the first time for the isolation of recombinant scFv antibody against N-fixing bacteria. The results encourage the further investigation of recombinant scFv antibody for the development new immunoassays for rapider and simpler detection of other bacteria using in agriculture as well as for studying in plant-microbe interaction.

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