GENETIC DIVERSITY OF STREPTOCOCCUS SUIS THAI ISOLATES AND CHARACTERIZATION OF ARGININE DEIMINASE

BY

MISS KRISSANA MANEERAT

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF THE
DOCTOR OF PHILOSOPHY
(BIOMEDICAL SCIENCES)

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ABSTRACT

Streptococcus suis (*S. suis*) is an important zoonotic bacterium causing life threatening diseases in piglets and humans. *S. suis* isolates from various Western countries as well as those from China and Vietnam have been previously characterized. At the commencement of this thesis, the genetic characteristics and relationship between *S. suis* strains isolated from both humans and pigs in Thailand were unknown. In this study, a total of 245 *S. suis* isolates were collected from both human cases (epidemic and sporadic) and pigs (diseased and healthy) in Thailand. *S. suis* were identified by conventional biochemical tests and PCR targeting 16S *rDNA* and *gdh* genes. Thirty-six isolates were identified as *S. suis* serotype 2 (*SS2*) based on serotyping and the *cps2*-PCR. Among the remaining 209 non-*SS2* isolates, the serotype was untypable for the majority of them (67.7%). For the known serotypes, serotype 22 was the most common among the 20 presented serotypes (9.6%). All of *S. suis* isolates were tested for the presence of six virulence-associated genes *i. e.,*

arginine deiminase (*arcA*), 38-kDa protein and protective antigen (*bay046*), extracellular protein factor (*epf*), hyaluronidase (*hyl*), muramidase-released protein (*mrp*) and suilysin (*sly*). The genetic diversities of these isolates were characterized by random amplified polymorphic DNA (RAPD) PCR. For the *SS2* isolates, multilocus sequence typing (MLST) analysis was also performed as an additional genotyping method. Four virulence-associated gene patterns (VAGP1 to 4) were obtained and the majority of isolates (32/36) carried all tested genes (VAGP1). Each of the three OPB primers used provided four patterns designated RAPD-A to RAPD-D. Furthermore, MLST analysis could also distinguish the 36 isolates into four sequence types (STs) *i. e.,* ST1 (n = 32), ST104 (n = 2), ST233 (n = 1) and a newly identified ST, ST336 (n = 1). Dendrogram constructions based on RAPD patterns indicated that *SS2* isolates from Thailand could be divided into four groups and the characteristics of the individual groups were in complete agreement with the virulence gene profiles and STs. The majority (32/36) of *SS2* isolates recovered from diseased pigs, slaughterhouse pigs and human patients could be classified into a single group (VAGP1, RAPD-A and ST1). This genetic information strongly suggests the transmission of *SS2* isolates from pigs to humans in Thailand.

Non-*SS2* isolates carried 13 VAGPs (excluding VAGP3). The 69 non-*SS2* isolates had both *arcA* and *bay046* genes (VAGP11). The phylogenic trees of non-SS2 *S. suis* isolates constructed from RAPD PCR banding patterns indicated that they were highly diverse. However, the genetic relationship between non-*SS2* isolates carrying VAGP1–4 was found to relate to the *SS2* isolates carrying the same VAGP indicating that a minority of non-*SS2* isolates carried the potential to transmit to human. This study is the first report for genetic characteristics of strains from Thailand and to elucidate the genetic relationship among *S. suis* isolates from human and pig origins.

ArcA of *S. suis* is one of the putative virulence factors. It could play an important role to facilitate intracellular survival of *S. suis* under acidic conditions. However, the biological properties and function of *SS2* ArcA have not yet been fully elucidated. This study revealed physical characteristics and enzyme kinetic properties of *SS2* ArcA. The *SS2 arcA* was cloned into *Escherichia coli* (*E. coli*) and expressed as a soluble protein under T7 RNA polymerase/promoter system. Recombinant *SS2*

ArcA (r*SS2* ArcA) was successfully purified using Ni-NTA affinity chromatography with EDTA gradient elution, yielding a 47 kDa protein. Multiple sequence alignment of *SS2-arcA* deduced-amino acid sequence revealed that it contained a highly conserved catalytic triad (Cys399-His273-Glu218) and had a high sequence similarity with that of *S. pyogenes* (89.98%). Enzymatic activity assay demonstrated that the r*SS2* ArcA was functionally active protein. The optimum temperature and pH of r*SS2* ArcA and crude native ArcA were 42 ºC and 7.2, respectively. The r*SS2* ArcA and crude native *SS2* ArcA were stable at 4 ºC and 25 ºC for 3 h but stability of crude native ArcA could be exceed to 30 ºC for 3 h. The pH stability and dependency tests suggested that the r*SS2* ArcA and crude native *SS2* ArcA were functionally active in acidic conditions. Enzyme kinetic studies demonstrated that the specific activity of r*SS2* ArcA was 16.00 U/mg and L-arginine substrate binding affinity (K_m) value was 0.058 mM. Whilst, specific activity and K_m values of crude native *SS2* ArcA were 0.23 U/mg and 0.157 mM, respectively. Compared to their orthologous proteins, the r*SS2*-ArcA exhibited a weak binding affinity with a common ArcA inhibitor, L-canavanine and L-NIO; therefore, neither r*SS2* nor crude native ArcAs were completely inactivated by these inhibitors. These results suggested that the inactivation mechanism of *SS2* ArcA might be distinct from other ArcA. Furthermore, the partial inactivation of ArcA significantly impaired the viability and growth of *SS2* at pH 4.0, 6.0 and 7.5 indicating the significant role of *SS2* ArcA for the survival of *SS2* under acidic conditions.

Keywords: *Streptococcus suis* (*S. suis*), virulence genes, random amplified polymorphic DNA (RAPD), multilocus sequence typing (MLST), arginine deiminase (ArcA), enzyme kinetic, physical properties

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LIST OF ABBREVIATIONS

Symbol/Abbreviation	Terms
%	Percent
°C	Degree(s) Celcius
× g	Gravitational acceleration
α	Alpha
µg	Microgram(s)
µl	Microliter(s)
µM	Micromolar(s)
[S]	Substrate concentration
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
Amp	Ampicillin
AP	Alkaline phosphatase
Arg	Arginine
ATP	Adenosine triphosphate
BA	Blood agar
bp	Base pair(s)
BSA	Bovine serum albumin
ca.	Approximately
CFU	Colony forming unit(s)
CO_2	Carbon dioxide
Cys	Cysteine
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
DW	Distilled water
EDTA	Ethylenediamine tetraacetic acid

LIST OF ABBREVIATIONS (Cont.)

Symbol/Abbreviation	Terms
ELISA	Enzyme linked-immunosorbent assay
e. g.	Exempli gratia (for example)
et al.	*et alli* (and all)
etc.	Et cetra (and the others, and other things, or and the rest)
FBS	Fetal Bovine serum
G	Gram(s)
Glu	Glutamic acid
H	Hour(s)
His	Histidine
i. e.	Id est (that is, or in other words)
IC_{50}	The half maximal inhibitory concentration
K_i	Equilibrium inhibition constant
K_m	Michaelis Menten constant
kDa	Kilodalton(s)
LB	Luria Bertani
l	Liter(s)
mM	Millimolar(s)
M	Molar(s)
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)
NBT	Nitroblue-tetrazolium salt
NCBI	National Center for Biotechnology Information
Ni-NTA	Nickle-nitrilotriacetic acid

LIST OF ABBREVIATIONS (Cont.)

Symbol/Abbreviation	Terms
nm	Nanometer (s)
nt	Nucleotide(s)
OD	Optical density (-ies)
ORF	Open reading frame
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
rpm	Rounds per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	Second(s)
sp.	Species
ssp.	Subspecies
TE	Tris-EDTA buffer
TEMED	N, N, N', N',-tetramethyl-ethylenediamine
Tween 20	Polyoxyethylene-sorbitan monolaurate
U	Unit(s)
UDW	Ultrapure distilled water
UV	Ultraviolet
V_{max}	Maximal velocity
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1
INTRODUCTION

Streptococcus suis (*S. suis*) is a facultative anaerobe gram-positive cocci. This bacterium is the important swine pathogen, particularly in piglets and young fattening pigs. Swine infections cause sepsis, meningitis, endocarditis, arthritis and pneumonia.[1,2] The natural habitat of *S. suis* is upper respiratory tract of adult pigs, particularly the tonsils and nasal cavity. *S. suis* is an important pathogen in newborn piglets and fattening pigs as well as adult pigs that have been raised in poor hygienic condition. Moreover, this organism is considered to be zoonotic agent of humans. The bacteria often mediate interspecies transmission to infect humans causing sepsis, meningitis, endocarditis, severe arthritis and septic shock with high case mortality rate or, to the minimum, irreversible deafness among the survivors. To date, *S. suis* can be classified into 35 serotypes based on serological differences of their polysaccharide capsules. *S. suis* serotype 2 (*SS2*) is the most prevalent serotype associated with diseases in pigs followed by serotypes 9 and 1, respectively.[3] *SS2* is the major causes of zoonotic infection in human. Serotypes 1, 4, 14 and 16 *S. suis* isolates had been rarely reported to associate with human infection.[4] In Thailand, *S. suis* zoonotic infection was first reported in 1987.[5] From 1987 to 2001, Thailand experienced only few sporadic cases.[6] The largest outbreak *Streptococcosis* in Thailand occurred during April to May 2007 due to the consumption of contaminated pork.

Apart from the capsular polysaccharide (CPS), other putative virulence candidates, such as an extracellular protein factor (EF, encoded by the *epf* gene), a muramidase-released protein (MRP, encoded by the *mrp* gene), the suilysin (SLY, encoded by the *sly* gene), adhesins, an arginine deiminase (ArcA), a 38 kDa BAY046 protein (encoded by the *bay046* gene) and a hyaluronidase (encoded by the *hyl* gene) have been suggested. However, their roles in the pathogenesis of human and swine infections remain poorly understood.[7–12] Genotyping of *SS2* isolates has been achieved using various methods including random amplified polymorphic DNA (RAPD)

PCR with the OPB7, OPB10 and OPB17 primers and multilocus sequence typing (MLST).[13–15] Diversity of the isolates from North America and Europe have been previously studied, and all isolates possessing an $MRP^{+}EF^{+}SLY^{+}$ phenotype were found to cluster in a single RAPD fingerprint, regardless of their geographical origins.[13] Consequently, *S. suis* strains with the similar phenotypic characteristics from both North America and Europe possibly originate from a common ancestor.[13,14] Previous reports have demonstrated that *S. suis* sequence type (ST) 1 is a highly evolved clone that predominates in human and pig infections in both Asia and Europe.[16] *S. suis* ST1 strains have also been associated with invasive diseases, such as meningitis, in both swine and humans in Thailand and Vietnam.[17,18] More recently, a new sequence type, ST7, was reported as the cause of a Chinese outbreak in 2005.[19] Many patients affected by *S. suis* ST7 strains developed streptococcal toxic shock-like syndrome (STSLS), resulting in a high mortality rate.[19] However, this clinical manifestation was also found in humans infected with ST1 strains in Vietnam,[18] but was rarely reported in patients infected with *S. suis* ST1 strains in Thailand.[20] Interestingly, and differently from Europe and Asia, most North American *S. suis* strains (including those isolated from human patients) belong to either ST25 or ST28.[21] In addition, new STs associated with human infections have been recently reported as ST104 and ST20 in humans from Thailand and the Netherlands, respectively.[16,17] Although some characteristics of Thai *SS2* strains have been studied, particularly those isolated from human patients,[17] the genetic relationships among *S. suis* isolates from both pigs and humans in Thailand have not yet been addressed. Therefore, it was important to investigate the virulence gene profiles and genetic relatedness among isolates from pigs (diseased and healthy) and human patients (epidemic and sporadic) using RAPD fingerprinting and MLST analysis.

Recently, two dimentional electrophoresis followed by immunoblot assay have been employed to identify *in vivo* expressed antigens of *SS2* among three infected species: humans, mice and pigs (Srimanote *et al.*, unpublished results). The obtained data showed that *SS2* arginine deiminase (ArcA) was one of the predominant *in vivo* expressed antigen during infection (Srimanote *et al.*,

unpublished results and Jing *et al*.[22]). Therefore, the *SS2* ArcA might have a presumptive role in the bacterial pathogenicity and might be one of the virulence-associated factors.

Arginine deiminase system (ADS) in prokaryote consists of three major enzymes, arginine deiminase (ArcA or ADI), ornithine transcarbamylase (ArcB) and carbamate kinase (ArcC). ArcA hydrolyzes L-arginine substrate to be L-citrulline and ammonia (NH_3). ArcB further converts L-citrulline to be ornithine and carbamoyl phosphate. Finally, the carbamoyl phosphate is further catalyzed by ArcC to be hydrogen carbonate, ATP and another molecule of NH_3.[23] Therefore, the ADS yields two NH_3 molecules as the final products. The ADS has also been detected in mycoplasmas, halobacteria, *Pseudomonas* sp., *Bacillus* sp. and a number of lactic acid and dental bacteria.[23] The NH_3 produced by ADS or urease could provide substantially longer survival of many oral streptococci in acidic environments.[24,25] These oral bacteria could rise the surrounding pH *via* ADS, by neutralizing the acidity derived from sugar metabolism thus increasing the cariogenicity followed the oral biofilms formation.[24,26,27] Moreover, the NH_3 production in *Pseudomonas via* ADS has been shown as a possible way to neutralize acidic pH.[28,29] In addition, a *S. pyogenes* mutant lacking ADS activity was unable to survive under acidic conditions in the presence of L-arginine that further influenced invasion and intracellular survival abilities of bacteria.[30] Furthermore, recent data indicated that both NH_3 and ATP productions of ADS are essential for streptococci adaptation in acidic environment extra- and intracellularly.[31]

Interestingly, ArcA has been shown to inhibit nitric oxide (NO) synthesis by depletion of plasma arginine resulting protection of mice from the effects of tumor necrosis factor α and endotoxin.[32] Unlike the prokaryotic system, there is no ADS in higher eukaryotic system, raising to the potential of ArcA as a biomolecular target for bacterial treatment.[33] More importantly, ArcA of prokaryotes and inducible nitric oxide synthase (iNOS) of eukaryotic cells share the same L-arginine substrate. Therefore, the presence of ArcA can deplete arginine level leading to interference of iNOS production and immune defense function by NO system. These have been supported by the iNOS-derived production of NO impairment in the murine innate

immunity in subcutaneous tissues by depleting arginine using *S. pyogenes* ADI pathway.[31]

It has been found that *SS2* ArcA could be regulated by temperature. Shifting temperature from either 32 ºC or 37 ºC to 42 ºC resulted in higher expression of ArcA.[9] In addition, the survival of ArcA-deficient noncapsulated *S. suis* in epithelial cell was significantly reduced at acidic pH condition.[28] Previous results also showed that the ADS enabled *SS2* to overcome oxygen and nutrient starvation and to tolerate acidic environments.[34] These findings highlighted the fact that *SS2* ArcA was likely to contribute to *SS2* survival in different niches. However, the biological characteristics of *SS2* ArcA have not yet been elucidated. Therefore, this study aims to characterize biological and physical properties and enzyme kinetics of *SS2* ArcA. These results could provide a better insight on the role of ArcA in *S. suis* pathogenesis.

CHAPTER 2
OBJECTIVES

2.1 Primary objective

The main objective of this research is to identify the role of ArcA in *S. suis* pathogenesis and determine of the prevalence of *arcA* gene and the genetic diversity of *S. suis* Thai isolates.

2.2 Specific objectives

2.2.1 To determine the prevalence of *arcA* and the other *S. suis* virulence genes in human and pig *S. suis* by simple and multiplex PCR.

2.2.2 To identify the genetic diversity of *S. suis* Thai isolates from pigs (diseased and healthy) and humans (epidemic and sporadic) by RAPD-PCR

2.2.3 To compare the virulence factor gene profile and genetic diversity of *SS2* and non-*SS2* isolates

2.2.4 To construct recombinant *E. coli* clone carrying *SS2* arginine deiminase coding-sequence (r*SS2 arcA*) using prokaryotic expression system

2.2.5 To express and purify soluble r*SS2* ArcA

2.2.6 To determine biological and physical properties of purified r*SS2* ArcA and crude native *SS2* ArcA

2.2.7 To determine r*SS2* ArcA enzymatic activity and its kinetic properties

2.2.8 To construct the *arcA*-negative mutant *SS2* derivative

2.2.9 To investigate the role of *SS2* ArcA in facilitating of *SS2* survival in acidic environment

CHAPTER 3
REVIEW OF LITERATURES

3.1 General background of *S. suis*

S. suis is facultative anaerobic gram-positive cocci that can be arranged in single cell, pairs and short chains under light microscopy. Following the 24 h incubation at 37 ºC, majority of the organism are alpha and beta hemolytic on sheep and horse red blood cells agar, respectively.[35] Based on cell surface antigenic determinants it is broadly classified to be related to Lancefield group D Streptococci, although their genetics are unrelated. Previously, *S. suis* was grouped into Lancefield groups R, S and T; however to date this nomenclature was restricted to *S. suis* strains belonging to only capsular serotypes 2, 1 and 15.[35] Therefore the Lancefield classification for this organism should be avoided.

This organism was identified by the inability to tolerate 6.5% sodium chloride (NaCl) and bile esculin, although, *S. suis* can hydrolyse esculin. Majority of them produce acid from trehalose and salicin. A rare *S. suis* isolate may be negative to trehalose or salicin but has never provided the negative results to both tests. Negative result for Voges-Proskauer (VP; acetoin production) test is an essential character to differentiate this organism from *S. bovis.* The additional biochemical test results for *S. suis* identification are positive for arginine dihydrolase and productions of acid from lactose, raffinose and glycerol. In addition, *S. suis* provides negative results to mannitol and sorbitol fermentations[36] (**Table 3.1**). Moreover, *S. suis* is often misidentified as alpha-hemolytic viridians streptococci, *Enterococcus faecalis* and *S. pneumoniae* (rare *S. suis* isolates are optochin susceptible) and other non-ABD group streptococci. The API20 STREP (Biomerieux®, France), commercial biochemical test based identification system, for this organism is validated for identification of only serotypes 1 and 2 *S. suis*.[37–41]

Table 3.1 Biochemical test results for *S. suis* identification[42]

Test	% of positive isolates					
	Serotype 2			Other serotypes		
	Total (n = 59)	Diseased pigs	Healthy pigs	Total (n = 59)	Diseased pigs	Healthy pigs
VP	0	0	0	9.1	20.0	5.9
Hippurate	0	0	0	27.3	40.0	23.5
Esculin	100.0	100.0	100.0	100.0	100.0	100.0
Pyrridonylarylamidase	0	0	0	18.2	40.0	23.5
α-galactosidase	24.3	83.3	12.95	80.0	35.3	0
ß-glucoronidase	73.0	100.0	67.7	86.4	40.0	100.0
ß-galactosidase	11.8	0	0	0	0	0
Alkaline phosphatase	16.2	0	19.4	45.5	0	58.8
Leucine aminopeptidase	86.5	100.0	83.90	60.0	100.0	0
Arginine dihydrolase	32.0	50.0	29.0	36.4	0	47.1
Ribose	16.2	0	19.4	40.9	0	52.9
Arabinose	5.4	0	6.5	9.1	0	11.8
Mannose	0	0	0	40.9	40.0	41.2
Sorbitol	2.7	0	3.2	40.9	0	52.9
Lactose	100.0	100.0	100.0	77.3	60.0	82.4
Trehalose	100.0	100.0	100.0	100.0	100.0	100.0
Inulin	21.6	33.3	19.4	50.0	60.0	47.1
Raffinose	64.9	83.3	61.3	77.3	80.0	76.5
Amygdalin	97.3	100.0	96.8	90.9	60.0	100.0
Glycerol	97.3	100.0	96.8	77.3	40.0	88.2

As a result of other biochemical tests used for *S. suis* identification give variable outcomes (**Table 3.1**), therefore, for conclusive identification, the molecular techniques are essential. Conventional PCR based on detection of *S. suis* 16S *rDNA* and glutamate dehydrogenase gene (*gdh*) is highly recommmended.[43] Consequently, PCR identification should be maintained in laboratories situated in regions where *S. suis* infection is problematic.[43] Following the species identification, *S. suis* can then be further classified for their capsular serotypes using serotype specific antisera. However, this can be done only for encapsulated isolates. It was found that only less than 5% of *S. suis* isolates recovered from diseased animals were not encapsulated, whereas, most of healthy pig *S. suis* isolates were not encapsulated.[35,44]

The natural habitat of *S. suis* is the upper respiratory tract, particularly the palatine tonsils and nasal cavities, genital and alimentary tracts of pigs.[8] The highly pathogenic *SS2* isolates colonize the palatine tonsils of clinically ill as well as apparently healthy pigs. *S. suis* asymptomatic carriage pigs are the reservoir and directly spread bacteria to other pigs in herds *via* nasal and oral-fecal routes.[45,46] Fomites, such as manure covered work boots and needles, also have been shown to be the vector for *S. suis* transmission.[47] Moreover, *S. suis* is highly tolerate in the environment. This bacterium could survive in water for 10 min and 2 h at 60 °C and 50 °C, respectively. *S. suis* also survived in carcasses for up to six weeks at 4 °C and up to one month in dust. Also, *S. suis* could be recovered from feces for more than three months at 0 °C.[48] Moreover, this bacterium survived in feces for 10 days at 9 °C, and up to 8 days at 22–25 °C.[49]

3.2 Clinical features

In swine, clinical signs can be varied from herd to herd, depending on virulence of the causative agent, health status and rearing condition. Pigs with pre-acute *S. suis* infection may die suddently or within hours after the onset of clinical signs.[50] In acute form of the disease, clinical signs begin with high fever (up to 42 °C), depression, anorexia and lassitude, followed by ataxia. Sometimes, they coordinated with neurological signs such as tremors, opisthotonus, blindness, hearing loss,

paddling, paralysis, dyspnea, convulsions, nystagmus, arthritis, lameness, erythema and/or abortion. The predominant signs of sickness of serotype 1 *S. suis* infection in 10- to 14-day-old piglets were becoming rough coated, fever and joint swelling.[51] The ultimate outcomes of *S. suis* infection are broad ranging from asymptomatic carriage to chronic disease and death. Lameness and/or residual central nervous system signs may be apparent in chronic disease. During *S. suis* outbreaks multiple strains of the same or different serotypes were often identified within an afflicted pigs. However, no significant differences in the clinical signs were observed in pigs infected with single or multiple serotypes.[52]

S. suis infection in human is a result of direct contact with infected swine or their products. In North American and European countries, the most common sign of human symptom is meningitis. However, arthritis, endocarditis, peritonitis, pneumonia and septic shock with multiple organ failure are also commonly reported. After treatment, hearing loss, ataxia, coma, neck stiffness, petechiae and facial paralysis often occur. Hearing loss is reported to be the most dominant clinical feature appeared following the recovery from purulent meningitis, whereas, death often follows septic shock.[53] The lesser common manifestation of infections in those countries include acute and subacute endocarditis, acute pyogenic arthritis, endophthalmitis and uveitis, spondylodiscitis, brain stem ophthalmoplegia and epidural abscess.[9,34,54–56] Recently, the distribution of clinical features of *S. suis* isolated from humans during 2002 to 2013[57] showed that serotypes 2 and 14 *S. suis* are involving in a high rate cases developing meningitis (50–70%). Moreover, these serotypes cause the septicemia in about 20–25% of cases. Other serotypes such as serotypes 4 and 21 were rarely isolated from meningitis patients, serotype 24 was collected from sepsis case. Serotypes 5 and 16 were involved in peritonitis patients.

A retrospective study at Chiang Mai University Hospital in Thailand during May 2000 to December 2002 revealed that 16 from 41 cases were endocarditis, whereas other 13 were meningitis, indicating unlike other countries, *S. suis* endocarditis was the most predominant clinical sign.[58] In addition, the data from Chulalongkorn University Hospital from January 1, 1997 to May 31, 2002 reported 12 meningitis patients with other neurological complications including deafness (33.3%),

subclinical sensory neutral hearing loss (16.7%), vestibular dysfunction (8.3%) and cerebritis/ventriculitis (8.3%). Thus, the patients with *S. suis* meningitis in Thailand suffered with subclinical hearing loss similar to that of Vietnamese meningitis patients (66.4%).[18] Recently, Kerdsin *et al*.[17] reported that approximately 60% and 35% of 165 human cases of *SS2* infection during 2006 to 2008 showed clinical manifestation as meningitis and sepsis, respectively. Moreover, among these cases hearing loss were reduced to only 21.5%.

3.3 Treatment and prevention

S. suis is susceptible to currently used ß-lactam antibiotics, including penicillin, ampicillin, amoxicillin and cephalosporin. Penicillin G is the mainstay for treatment or prophylactic control of *S. suis* infection. In addition, ceftriaxone is used for treatment of *S. suis* infected meningitis human patients and sometime, ampicillin and chloramphenicol can be combined with an aminoglycoside for treating *S. suis* infection in human.[18] Currently, *S. suis* infection in animals and human are controlled by the used of antibiotics as prophylactic and therapeutic agents. However recently, antibiotic-resistant *S. suis* isolates have been increasingly reported and rose the public health concern. Many vaccines including live attenuated and killed *S. suis* and recombinant putative virulence factor had been developed for infection control. However, none of them could provide broad spectrum protection across heterologous strains and serotypes of *S. suis* infections. The failure on vaccine development is dued mainly to the shortage of information on pathogenesis and the role of virulence factors of *S. suis*.[59]

3.4 Epidemiology

Infections caused by *S. suis* represent significant economic problems in countries with intensive swine industries, such as Spain, Australia, Germany, Belgium, the Netherlands and the United Kingdom. Moreover, this organism could be found in other animals, such as horses, dogs and cats. Among 35 different serotypes, *SS2* is

the most important serotype to affect pigs and humans. This serotype is also responsible for more severe forms of infections in both swine and human worldwide.[53]

S. suis infections in most of weanling piglets are caused by *SS2*. This serotype also associates with the disease in fattening piglets and neonates.[59] Among North American and Asian countries, *SS2* pig infection is the most virulent and prevalent.[53,60,61] The serotype 1 *S. suis* is also often found to associate with diseases in piglets and older pigs in the United Kingdom and the United States.[51,62] The other prevalent serotype reported in United Kingdom is serotype 14.[63,64] On the other hands, piglets infection with serotype 9 *S. suis* is very common for Australia and continental European countries such as Belgium, Germany, Spain and The Netherlands.[61,64,65]

The first human transmission of *S. suis* was reported in 1968 from Denmark followed by an endocarditis case in Canada.[45] Human *S. suis* infections happened mostly on the individuals who worked in direct contact with infected swine or in the industrial processing of pork products. Although human zoonotic *S. suis* infection worldwide is almost conclusive case by serotype 2 but serotypes 1 and 14. *S suis* strains also have been reported at very low frequency as causative agents.[66,67] From 1968 to 2004, 200 human infections were reported worldwide, involving only sporadic case.[68,69] To date, human infections in Asia have been reported mostly from China, Thailand and Vietnam accounting for 83.6% of annual human *S. suis* zoonotic diseases worldwide.[57] Majority of human cases during 1998 to 2005 were reported from China,[70] moreover, almost all cases (39 patients) reported during 1989 to 1999 died with the toxic shock syndrome and meningitis from *S. suis* infection.[53] From mid-July to the end of August in 2005, the largest outbreak of *S. suis* human infection occurred in Sichuan Province of China. It involved 204 human cases and 38 deaths.[53] The high mortality rate is a consequence of lacking of prompt or timely treatment.[53] At the same period, sporadic *S. suis* human cases were also reported in other regions of the country, including four cases with one death in Guangdong Province, two in Jiangsu Province and ten in Hong Kong.[19,70] This human outbreak was in concomitantly to the large outbreak that involved infection of

80,000 pigs.[19] It was proved that 97% of patients had direct contact with sick pigs including slaughtering, having skin cuts while contact the sick pigs and/or consumption of improper cooked pork from sick pigs. Person-to-person transmission is not occurred in these outbreaks. To date, the information obtained from all epidemiological surveys of the outbreaks in China indicated that direct contact with sick pigs is the key factor for *S. suis* infection.[70] The global epidemiology of *S. suis* infection in humans is summarized in **Figure 3.1**.

In Thailand, *Streptococcosis* caused by *S. suis* was firstly reported in 1987 in Bangkok.[71] From 1994 to 2001, there were at least 21 sporadic cases in human reported. However, from January 1999 to August 2000, 10 of the cases were reported from Lampoon Province alone. All of the reported cases were adult males (40–50 years) with a risk factor of alcohol drinking. Seven of them had the history of consuming raw pork.[58] The descriptive prospective observation of distribution of *Streptococcosis* in Lampoon Province from July, 2001 to July, 2003, indicated that from 28 patients which had been previously diagnosed with *S. viridian* infection, 19 of them were actually infected with *S. suis*. Three cases developed deafness and seven were dead (37% mortality rate). All of these patients had history of raw pork consumption. Information from public health authority indicated that *S. suis* infection could be found throughout the year with the increasing incidence during May to August.[72] From the retrospective study of 12 *S. suis* infected patients during 1997 to 2002 indicated that the dominant symptom was meningitis similar to that of reported in patients in Hong Kong and the Netherland. During May, 2000 to December, 2002 there were at least 41 human cases of *S. suis* infection admitted to Chiang Mai Hospital, and all of them died.[58] Among these, three had contact with sick pigs and one had history of eating raw beef. The largest *S. suis* outbreak in Thailand occurred in Phu Sang Sub-District, Phayao Province in April 2007. It involved more than 300 patients and the outbreak was associated with the consumption of raw pork blood and meat. The fatality rate was 20% and 20% of the infected individuals developed two-ear permanent hearing loss. The retrospective study of *SS2* infection in Thai patients during 2006 to 2008 was conducted.[17] The data showed that 165 patients infected with this bacterium were frequently found in rainy season. The majority of

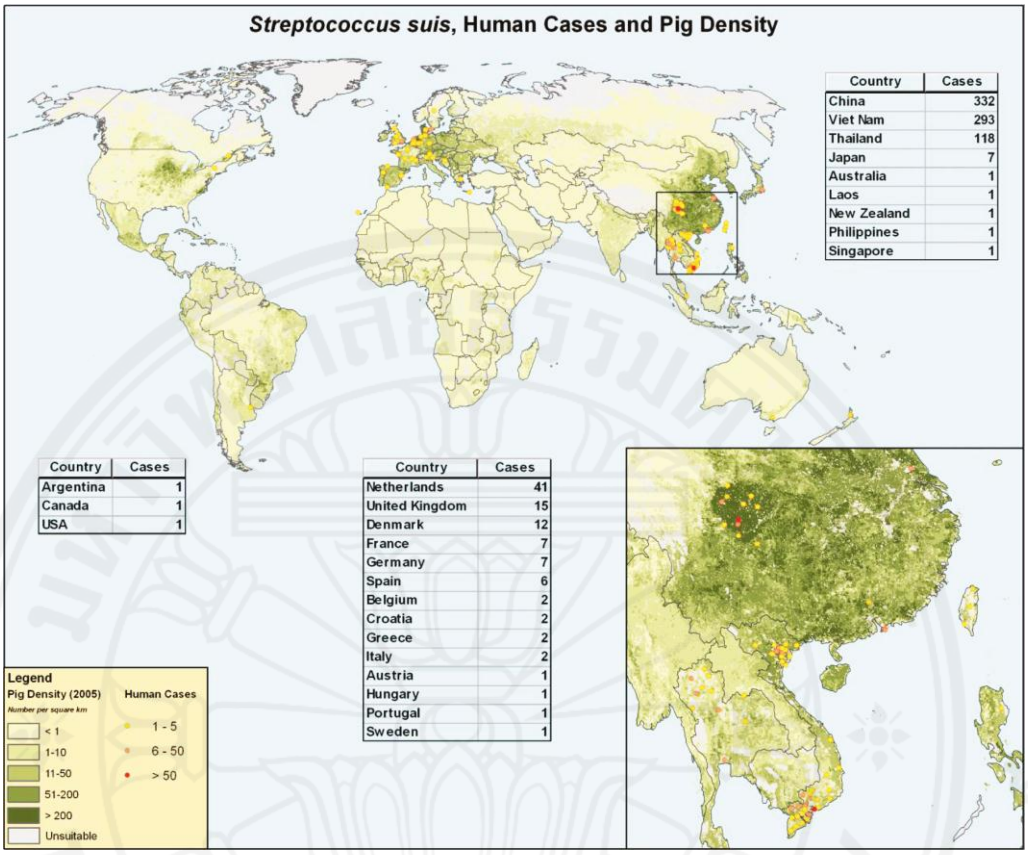


Figure 3.1 Global epidemiology of human *S. suis* cases with background pig density data[73]

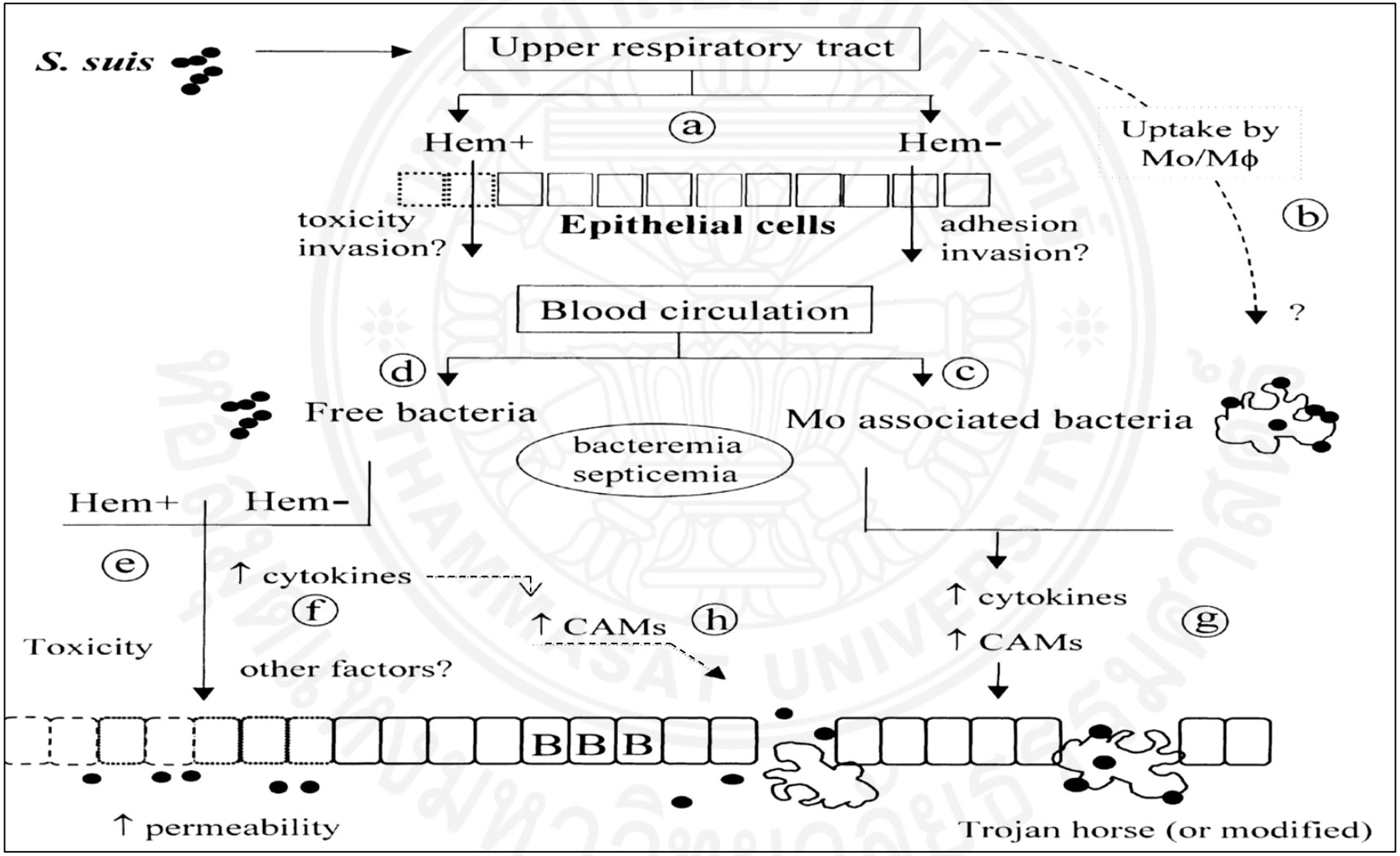
patients (82.4%) were found in the Northern provinces. Approximately 11.5%, 4.2% and 1.8% of patients were reported from Central plain, Northeast part and Eastern provinces, respectively. Interestingly, no case was reported from the Southern part of Thailand. The major clinical manifestations were meningitis and sepsis. The mortality rate was approximately 9.5%. During 2011 to 2013, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, reported 495 patients composed of 374 males (75.6%) and 121 females (24.4%) with 6.46% mortality rate. Most of these patients had history of raw pork consumption and the majority of patients were Northern provincial, especially Nakhon Sawan (124 cases, 25.1%) and Chiang Mai (94 cases, 19%). In 2014, *S. suis* human infection were 226 cases with 6.64% mortality rate (15 cases).[74] Recently, Ministry of Public Health reported 307 patients with 4.89% mortality rate (15 cases) during January 1, 2015 to October 30,

2015. The majority of patients came from Nakhon Sawan Province (53 cases, 17.26%), Nan Province (48 cases, 15.64%), Nakhon Ratchasima Province (45 cases, 14.66%).[74]

3.5 Pathogenesis

The pathogenesis of *S. suis* infections is not well understood because of the presence of multiple serotypes and the differences in virulence among different strains of the same and different serotypes.[75] To date, almost all of *S. suis* pathogenesis studies have focused only on the *SS2* strains. The clinical symptoms in patients and pigs are suggested to be the outcomes of both the capability of the bacteria to cause the disease and immunopathology caused by host immune response to the *S. suis*.[76] Although the routes of entry in human (skin abrasions and gastrointestinal mucosa) and pig (respiratory mucosa) are different, the pathogenic mechanism is believed to be similar.[77] The proposed pathogenic mechanisms are depicted in **Figure 3.2**.[8]

After successful colonization of tonsil or nasal cavity combined with the susceptible conditions of the pigs such as very young age, weaning, poor hygiene rearing condition and stress, *S. suis* spread further to colonize palatine tonsil and respiratory epithelial cells. The bacteria then gain the access to efferent blood supply or lymphatic circulations, resulting in bacteremia or septicemia. To date, the mechanism whereby *S. suis* translocate from epithelial cells to underlying tissues of the swine respiratory tract and human gastrointestinal tract and their microvasculature including their travelling to the target organs such as the CNS and ear has not yet elucidated. However, three different pathways of acquiring access to blood circulation were speculated. Firstly, hemolysin (suilysin) produced by *S. suis* may cytotoxic to both epithelial and endothelial cells and breached the integrity of lining mediated entering of hemolysin positive *S. suis* into blood stream. Secondly, suilysin negative strains may directly invade epithelial and endothelial cells by the unknown mechanism. Finally, it is also possible that the bacteria may be engulfed by respiratory macrophages and entered to the bloodstream within these cells. As soon as *S. suis* emerge in the blood circulation, they may survive as free bacteria and



(Please see overleaf for figure legend)

Figure 3.2 Summary of the proposed steps involved in the pathogenesis of meningitis due to *SS2*. Hem+: hemolysin-positive strains; Hem−: hemolysin-negative strains; Mo: monocytes; MØs: macrophages; CAMs: cell adhesion molecules; BBB: blood-brain barrier; CNS: central nervous system. Steps a and b show *S. suis* interactions with the epithelial cell layers of the upper respiratory tract (colonization) and access to blood circulation. Hem+ strains may use cell disruption (toxicity) and invasion to reach the bloodstream, while the mechanisms used by adhered Hem− strains are still uncertain (Step *a*). *S. suis* could also be directly uptaken by Mo/MØ and enter to the bloodstream within circulating cells (Step *b*). Steps c and d describe bacterial travelling in blood. This could be as Mo-associated (bound and/or intracellular) bacteria (Step *c*) or as free bacteria (Step *d*), resulting in bacteremia or septicemia. Steps e and h describe the possible mechanisms used by *S. suis* to cross the BBB. Free bacteria would enter the CNS after increasing BBB permeability, *via* direct cell toxicity (Hem+ strains; Step *e*), indirectly *via* local cytokine production (Step *f*) or *via* other unknown(s) mechanism(s). Local cytokine production could also increase CAMs expression and leukocyte migration, that in turn "open the door" to free bacterial trafficking (Step *h*). On the other side, Mo-associated bacteria would enter the CNS *via* the "Trojan horse" (bacteria inside cells) or "modified" Trojan horse (bacteria adhered to cells) mechanism favored by activated phagocyte cytokine release (Step *g*) (Reproduced from Gottschalk M, Segura M. 2000)[8]

cause septicemia or alternatively, they may be engulfed by monocytes in circulation. These cells do not kill bacteria; instead carry them to the capillary of central nervous system (Trojan horses). *S. suis* may then destroy the integrity of blood brain barrier (BBB) by direct cytotoxicity or mediating apoptosis and necrosis of brain microvascular endothelial cells (BMEC) and choroid plexus epithelial cells (CPEC) constituent of BBB. It has been shown that *S. suis* affects CPEC barrier function and integrity by the induction of apoptosis and necrosis.[77] Furthermore, it has been shown that *S. suis* induced rearrangement of the tight junction proteins ZO-1, occluding and claudin 1 resulting in loss of actin at apical surface and increasing in basolateral stress fiber formation. As a consequence the tight-juctions between the epithelial cells were impaired leading to leukocytes transmigration from the apical side to the basolateral side and increased movement of both free *S. suis* and monocytes/macrophages carrying *S. suis* (Trojan horse) in the opposite direction. Thus, an inflammatory response at BMEC and CPEC lead to invasion of CNS by *S. suis* and resulted in meningitis, damaging of neurons and other clinical signs of neurological diseases.[78,79]

3.6 Putative virulence candidates

Many of the virulence candidates of *SS2* were extensively studied, however, little is known regarding to their role in pathogenesis. The putative virulence determinants of *SS2* include the capsular polysaccharide (CPS), muramidase-released protein (MRP) and extracellular protein factor (EF), the hemolysin (suilysin), several adhesins, hyaluronate lyase (Hyl) and arginine deiminase (ArcA or ADI).

3.6.1 Capsular polysaccharide (CPS)

The CPS of *SS2* composed of glucose, galactose, *N*-acetylglucosamine, rhamnose and sialic acid in a molecular ratio of 1:3:1:1:1.[80] It is only the proven virulence factor of *S. suis* identified to date and confirmed to have a major role on protection of organism from phagocytosis. The standard serological

classification of *S. suis* strains is based on identification of the presence of specific epitopes on their CPS.[40,41,81] The severity of the disease is varied among serotypes, suggesting its contribution to the virulence. It had been demonstrated that only serotypes 1 and 2 *S. suis* that harbor additional virulence factors are virulence in pig and human. Whereas, the respective serotypes which lack of other virulence determinants are avirulent.[82] Thus, this strongly suggested that capsule is not a sole virulence factor of *S. suis* based on site-directed mutagenesis, roles of capsule in *S. suis* resistance to phagocytosis was demonstrated.[83] The defect in CPS production resulted in increasing in *S. suis* cellular hydrophobicity and enhancing of *S. suis* phagocytosis by murine and porcine phagocytes.[83] Moreover unencapsulated isogenic mutants were avirulent in mouse and pig models compared to that of the wild type parents. In addition, encapsulation has been suggested to be down-regulated during colonization of epithelial cells and nasopharynx to facilitate adherence and colonization. On the other hand, the bacteria in the bloodstream capsule will be up-regulated to evade host immune system and promote interaction of bacteria with endothelial cells of the BBB.[84] In addition, it was shown that CPS influences the relative expression of Toll-like receptor 2 (TLR2) and CD14 mRNA; thus TLR activations may due to an indirect consequences of NF-kB and AP-1 transcription factors up-regulation *via* MCP-1 activation pathway.[55] MCP-1 has been known to regulate the recruitment of leukocytes to the sites of inflammation and mediate alteration of endothelial cell tight junction. Therefore, these may result in gaining access of bacteria to CNS, characteristic of *S. suis* infection.[85]

3.6.2 Muramidase-released protein (MRP) and extracellular protein factor (EF)

MRP and EF present only in diseased pig isolates and absent from most of healthy pig isolates. MRP is a 136-kDa protein. MRP predominate in protoplast supernatants and may be associated with peptidoglycan. The molecular weight of free MRP in the culture supernatants is smaller than that of protoplast-bound MRP. Molecular weight of MRP is diverged. Most of low pathogenic *S. suis* pig isolates carried smaller MRP (MRP*). MRP is homologous to M protein of group A Streptococci. M protein is peptidoglycan associated and facilitates bacteria to

phagocytosis resistance and mediates adherence of *S. suis* to epithelial cells. The M proteins also vary in their size and antigenic composition. Analysis of 136-kDa MRP showed that it has an anchor sequence similar to that of group A streptococci M-proteins. Therefore, MRP may involve in binding to host fibronectin similar to that of M protein.[86]

EF is a secreted 110-kDa protein that was detected exclusively in culture supernatant. However, its role in pathogenesis is unknown. The majority of strains isolated from disease pigs with meningoencephalitis, polyserositis and polyarthritis often produce EF. On the other hand, the organisms that cannot produce EF are often associated with mild disease. Size of EF produced by *S. suis* is heterogeneous. Analysis of the deduced amino acid sequence of EF derivative (EF^{*}) indicated that it possesses an additional short repeated amino acid sequence (~76 amino acids) resulting in the larger molecular weight.[87]

The association of the presence of MRP and EF to the virulence has been originally reported in *SS2*. Interestingly, the patient isolate carries MRP and EF^{*} was avirulence in pigs. The virulence of MRP and EF defective mutants were comparable to that of the wild type using new-born germfree piglet model. Association of MRP and EF to pathogenicity of *S. suis* was evident only in strains isolated from European countries. Most of pathogenic *S. suis* strain isolated in North America did not produce MRP and EF proteins.[88] These suggested that MRP and EF are not essential for *S. suis* pathogenesis.

3.6.3 Suilysin (Sly)

Sly is a secreted protein belonging to thiol-activated cytolysin (TACY) family of hemolysins. Members of this include several pore-forming toxins produced by gram-positive bacteria including streptolysin O (SLO), perfringolysin O (PFO), listeriolysin O (LLO) and pneumolysin (PLY).[89] The *sly* DNA sequence showed high similarity (99.5%) to the PLY toxin produced by *S. pneumoniae*. Sly is further classified based on its antigenic properties into subfamily of toxins known as cholesterol-binding toxins which forms transmembrane pores and it possesses multiple mechanism of action in other bacteria.[90] The *S. suis* Sly in culture supernatants has molecular size range from 54 to 65 kDa.[91,92] The role of Sly in

S. suis in pathogenesis remains unclear, however, it may involve in the lysis of cholesterol containing cytoplasmic membrane found in all eukaryotic cells. Recently, it was demonstrated that the *sly* is not present in all *S. suis* isolates, suggesting that Sly may not essential for *S. suis* pathogenesis.[93] The distribution of Sly in *S. suis* isolates is similar to that of MRP and EF. European *SS2* isolates carrying *mrp* and *epf* genes also harbor Sly, while the North American strains are hardly positive for EF, MRP and Sly. The *sly* gene was highly conserved among strain as 99.5% DNA sequence identity were observed between the North American strain (SX332) and the European strain (31533).[93] Due to the difference in G+C content of *sly* compared to the rest of the genome, it was indicated that *S. suis* acquired *sly* gene from horizontal gene transfer and thereafter spreading among *S. suis* isolates by homologous recombination.[94] Although, Sly mediated epithelial invasion and cell lysis *in vitro*,[95] Sly negative defined mutant was only marginally attenuated in porcine model.[93] Recently, it was reported that many strains of *S. suis* isolated from diseased pigs lack Sly but, Sly has never been reported in avirulent *S. suis* strains.

Importantly, MRP, EF and SLY have been considered to be virulence markers based on the mutation of these three genes that encoded each protein remain still had virulence similar to that of the wild type parent. It was suggested that the production of MRP, EF and SLY are not essential for *S. suis* pathogenesis. To date, MRP, EF and SLY are considered as virulence markers for detection of the virulence strains rather than the virulence factors.[12]

3.6.4 Hyaluronate lyase (Hyl)

Hyl of *S. suis* is a secreted protein that degrades hyaluronic acid (HA) into unsaturated disaccharides. HA is a linear glycosaminoglycan consisting of a polymer of a ß-1-4 linked N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) disaccharides. It is a major component of the extracellular matrix of tissues and body fluid as well as the capsule of microorganisms.[96] In general, a carbon source from disaccharide degradative product of Hyl offers nutrient for further catabolism during growth and colonization at the site of infection.[97–99] Hyl is produced by a variety of gram-positive and some gram-negative bacteria.[100] This enzyme may play a role in the early step of *S. suis* pathogenesis involving in establishment of bacteria at

mucosal or skins surfaces of humans and animals. Connective tissues showed decreasing in viscosity after depolymerization of HA by Hyl. Connective tissue integrity is decreased facilitating *S. suis* spreading further deep into those tissues. Alternatively, Hyl may promote adherence by degrading HA coated on the cell surface resulting in exposure of specific receptors for bacterial adhesin on the host cell surface.

Hyl has been found in streptococci of Lancefield groups A, B, C and G streptococci.[101,102] Virulence strains of *S. agalactiae*, group B Streptococci, caused invasive disease often secrete higher level of extracellular Hyl than asymptomatic disease isolates.[103,104] Signature tagged mutagenesis of *hyl* in *S. pneumoniae* genome indicated that it plays part in pneumococcal pneumonia, but it is dispensable in septicemia form of the infection.[105]

hyl gene from *S. suis* encodes for protein with a typical gram-positive signal peptide and cell wall associated LPXTG motif indicating its association with peptidoglycan on the cell surface. King and co-workers (2004)[106] demonstrated that only 29.4% of the *hyl*-positive isolates could produce Hyl with hyaluronidase activities regardless of their isolation origin (healthy pigs derived). The *hyl* gene of pig lethal strain P1/7 *S. suis* possesses a premature stop codon resulting in a production of truncated protein of 522 amino acids lacking of hyaluronidase activity. In contrast, a lesser virulence serotype 7 *S. suis* isolate harbors complete *hyl* (Genbank accession number AJ308328) and possesses hyaluronidase activities *in vitro*.[106] Taken together with the absence of Hyl activities in many virulent strains, it is suggested that Hyl is not an important virulence factor of *S. suis*.[106]

3.6.5 38-Kilodalton immunogenic and protective antigen (BAY046)

Okwumabua and Sharmila identified a novel *SS2* virulence factor, BAY046.[11] This 38 kDa protein appeared to be immunogenic in the infected individuals and could provide the protective immunity in pigs against lethal dose challenge of *SS2*. Southern hybridization analysis indicated that prevalence of *bay046* is high among diseased pig isolates and it distributes across in 31 serotypes of *S. suis* (excluding for serotypes 20, 26, 32 and 33). Western blot analysis showed that

BAY046 protein localizes on the surface of bacteria. However, the role of BAY046 in *S. suis* pathogenesis remains uncovered.[11]

3.6.6 Arginine metabolism and arginine deiminase (ArcA or ADI)

3.6.6.1 Arginine metabolism

Arginine is a vital amino acid precursor for biosyntheses of other amino acid and molecules *i. e.*, citrulline, ornithine, L-proline, glutamate, agmatine and urea as well as other biological modulators such as nitric oxide (NO) and polyamine. Moreover, one of arginine immediate degradation product, L-citrulline is the precursor for production of carbamoyl phosphate which is the substrate for biosynthesis of pyrimidine bases.[107] There are at least four enzymes for arginine degradation, arginase, arginine deiminase (ArcA or ADI), arginine decarboxylase and nitric oxide synthase (NOS). Arginase, found preferentially in eukaryote, converts L-arginine to urea and L-ornithine. This amino acid is then changed to L-proline for collagen and polyamines (*i. e.*, putrescine, spermidine and spermine) synthesis for cell division process. The second enzyme, ADI, found exclusively in bacteria and *Giardia intestinalis*, catalyzes L-arginine changed to be L-citrulline and ammonia (NH_3). The third enzyme, arginine decarboxylase is also prokaryotic enzyme involving in glutamate production and urea metabolism by conversion of L-arginine amino group.[107] It cleaves the carbon-carbon bonds in amino group to produce glutamate, agmatine and carbondioxide. The last enzyme, NOS is eukaryotic enzyme that metabolites L-arginine by oxidative deamination to produce nitric oxide (NO), citrulline and nicotinamide adenine diphosphate (NAD^+ or NADPH).[108] Upon activation eukaryotic cells such as, endothelial cells, macrophages and neuron can stimulate NOS production follows by synthesis of NO from L-arginine substrate resulting in vasodilatation, killing invading microorganism and non-adrenergic, non-cholinergic neurotransmission, respectively.[109]

3.6.6.2 Nitric oxide syntases (NOS) and nitric oxide (NO)

NOS have three isoforms, neuronal, endothelial and inducible NOS. Neuronal NOS (nNOS or NOS1) is constitutively presents in central and peripheral nervous systems, to produce NO that acts as a neurotransmitter. Endothelial NOS (eNOS or NOS3) is constitutively expressed in endothelium and

smooth muscle cell involving in cardiovascular homeostasis. It is also known that NO produced by eNOS act as a key mediator to modulate the activity of vascular endothelial growth factor, basic fibroblast growth factor and matrix metalloproteinases during angiogenesis of tumor.[110,111] Inducible NOS (iNOS or NOS2) is absent in cells in resting stage. *iNOS* gene is rapidly expressed in response to the extracellular stimuli, such as, proinflammatory cytokine, endotoxin and bacterial cell wall components. The NO produced by iNOS is generally 100-1000 times higher than that of nNOS and eNOS. However they have a shorter half-life.[108]

iNOS was originally identified and characterized in macrophages. Stimulation of macrophage by endotoxin or cytokines results in generation of NO to kill and inhibit the growth of invading microorganisms and neoplastic tissue.[112] Conversion of L-arginine to L-citrulline and NO by iNOS depends on the available of the L-arginine substrate and the cofactors. The catalysis involves two electron oxidation steps (**Figure 3.3**).

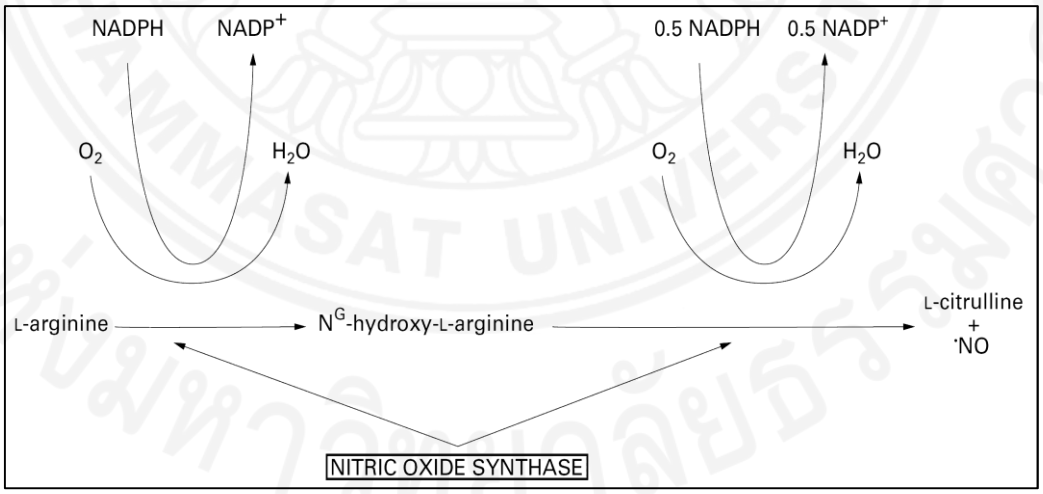


Figure 3.3 Conversion of L-arginine to L-citrulline plus nitric oxide as catalyzed by nitric oxide synthases (Reproduced from Burgner D, Rockett K, Kwiatkowski D. 1999)[108]

First, the guanidine nitrogens of L-arginine are hydroxylated in the presence of molecular dioxygen and NADPH to form N^G-hydroxyl-arginine. The

second step is a three electron oxidation that also required molecular dioxygen and NADPH to form L-citrulline and the free radical NO. NO shutdown bacterial DNA synthesis by causing double stranded breaks (DSBs) through ribonucleotide reductase 1/2 inhibitions thereby inhibit bacterial pathogen replication. Moreover, those resultant NO mediated oxidative damage are potentially harmful to the host cells. Meanwhile, the microorganisms have several mechanisms for protect themselves against NO mediated oxidative damage in host cells including L-arginine substrate competition by ADI or resistance to oxidative damage mediated by NO. Moreover, some bacteria carry superoxide and peroxide regulons for sensing the presence of NO and then produce superoxide dismutase and alkyl hydroperoxide reductase to damage it.[113]

ADI has L-arginine as substrate similar to NOS therefore it has ability to down-regulate NO production by precursor deprivation or substrate competition. Ability of ADI to deplete serum arginine and subsequently suppress LPS-induced NO production in macrophage were demonstrated.[32,112] Moreover anti-proliferative and anti-angiogenesis effect of ADI was also demonstrated as a consequence of arginine deprivation followed by lacking of NO-mediated angiogenesis growth factor production. These effects were reversible as they could be restored by supplement copious amount of arginine.[113,114] Moreover, recombinant ADI of *M. arginini* expressed and purified from eukaryotic system could selectively inhibit the growth of human T cells and T lymphoblastoid cell lines. The morphology of dying cells showed DNA fragmentation suggesting that ADI induced apoptotic cell death. In addition, ADI has been shown to inhibit NOS mediated NO synthesis resulting antiangiogenic effects and exert effects against tumor necrosis factor-α (TNF-α) which have been stimulated by endotoxin.[115]

3.6.6.3 Prokaryotic ADI

ADI belongs to the member of in hydrolases family, other names for ADI are arginine dihydrolase or citrulline iminase. Arginine deiminase system (ADS) presents in many prokaryotic organisms, including *Bacillus* sp., *Pseudomonas* sp., halobacteria, lactic acid bacteria and oral streptococci[23,24]. Moreover, ADI also has been discovered and characterized in archaea and anaerobic

eukaryotes, *G. intestinalis*.[116] However, it has not yet been reported in higher eukaryotes.[117] The ADI pathway consists of three major enzymes *i. e.,* arginine deiminase (ADI, EC 3. 5.3.6), ornithine transcarbamylase (OCT, EC 2.1.3.3) and carbamate kinase (CK, EC 2.7.2.2) (**Figure 3.4**). ADI hydrolyzes L-arginine to be L-citrulline and NH_3. L-citrulline is then converted to ornithine and carbamoyl phosphate by OCT. Finally, CK further catalyzes carbamoyl phosphate in the presence of ADP the reversible conversion to be carbon dioxide (CO_2) and NH_3. Usually, one mole of L-arginine metabolism provides one mole ATP, CO_2 and ornithine and two moles of NH_3 (**Figure 3.4**).[33] The genes encoding the ADS are generally organized as an operon. However, the genetic constitution and organization of operon can be varied depending on the species of the organisms. The operon is commonly composed of *arcA*, *arcB*, *arcC* and *arcD.* Some organisms have additional regulatory genes, aminopeptidase (*arcT*) and a transcriptional regulator of the Crp/Fnr family (*arcR*) or ArgR/AhrC family.[118]

ADI is known to be up-regulated under catabolic repression and subsequently promoted the survival of the organism under this condition. ADI also facilitated the growth of *P. aeruginosa* and *B. licheniformis* under the transition from aerobic to anaerobic condition.[119,120] Moreover, it was found that ADI activities were increased when oral streptococci and *Lactobacillus sakei* were grown under carbon catabolite repression (CCR). Furthermore, ADI activity was further elevated by addition of L-arginine to the culture medium.[121] Increasing in ADI activities followed by ammonia production also suggested to the mechanism for survival of oral streptococci and *S. pyogenes* under acidic stress.[24,30] Terminal amino acid sequencing analysis of the *S. pyogenes* ADI had identity to the previously characterized protein namely, streptococcal acid glycoprotein (SAGP). SAGP possesses 31.5 to 39.0% amino acid sequence identity to ADI from *M. hominis*, *M. arginini*, *P. putida* and *P. aeruginosa*.[121]

Cell extracts (CE) prepared from a wide range of *S. pyogenes* strains contained ADI and could inhibit phytohemagglutinin (PHA) mitogenic activity thereby it inhibits proliferation of human peripheral blood mononuclear cell (PBMC) *in vitro*.[121] Heating CE at 55 ºC for 10 min to destroy ADI

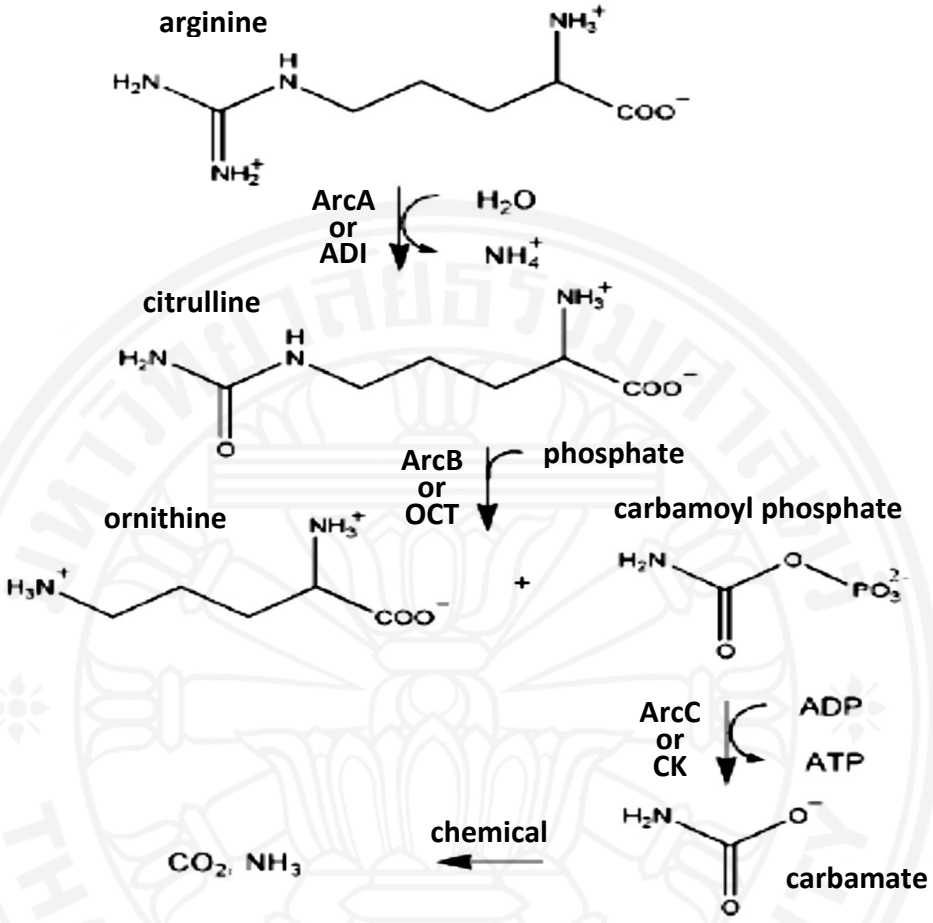


Figure 3.4 Arginine deiminase pathway[33]

activity could result in restoration of PHA mitogenicity on PBMC. Moreover, the similar outcome was observed by addition of natural substrate, L-arginine.

In *S. suis*, ADI or ArcA was found to be upregulated by shifting temperature up from 32 ºC to either 37 ºC or 42 ºC.[9] The survival of ArcA-deficient noncapsulated *S. suis* in epithelial cell was significantly reduced compared to that of wild type parent. Moreover, an *arcA* mutant lost their ability to survive in *in vitro* at low pH condition.[28] These findings highlighted the fact that ArcA might contribute to *S. suis* survival in different environments both *in vivo* and *in vitro*. However, the contribution of ArcA in *S. suis* pathogenesis including anti-mitogenic activity on stimulated PBMC and intracellular survival in macrophage has not yet been elucidated.

3.7 *S. suis* genotyping

3.7.1 Multilocus sequence typing (MLST)

MLST has potential role in studying the intra-species genetic relatedness. The principle of MLST based on PCR amplification of several housekeeping genes followed by DNA sequencing analysis of nucleotide polymorphisms. This method has shown a high degree of intra-species discriminatory power for many bacterial and fungal pathogens. A number of the selected housekeeping genes are varied among the species depending on the degree of discrimination desired.[122] MLST directly analyzes the DNA sequence variations in the targeted set of housekeeping genes and classify the tested strains into the sequence type (ST) according to their unique allelic profiles. Therefore, ST is an unambiguous descriptor or identity for the strain.[123] Isolates that share the same ST are considered as the same clone derived from one common ancestor. Thereafter, the relatedness of the individual ST is investigated by using eBURST software embedded in the MLST database, the closely related STs will be further assigned into clonal complexes (CC). Generally, CC is composed of a single predominant ST with a few other closely related ST(s). The eBURST analysis provides the additional overview of genetic relatedness, diversity and the emerging of the new clones in the individual geographical areas.[123,124]

For *S. suis* genotyping by MLST, seven housekeeping gene fragments, a 5-enolpyruvylshikimate 3-phosphate synthase (*aroA*), a 60 kDa chaperonine (*cpn60*), a peroxide resistance protein (*dpr*), a glucosekinase (*gki*), a DNA mismatch repair protein (*mutS*), a homologous recombination factor (*recA*) and an aspartokinase (*thrA*) are commonly used.[125,126] Initially, 92 STs from 294 *S. suis* isolates were obtained from diseased and asymptomatic pigs.[125] ST1 was the most common genotype and associated with isolates from meningitis, sepsis and arthritis while other STs were obtained from isolates which appeared to be lesser virulent. Currently, over 672 STs have been identified and deposit into *S. suis* MLST database (Available from http://ssuis.mlst.net/; Accessed on December 2, 2015) indicating the vast genetic diversity of *S. suis* strains.

Distribution of the most common ST(s) of *SS2* isolates from diseased pigs and patients worldwide during 2002 to 2013 are shown in **Figure 3.5**. It has been demonstrated that *S. suis* ST1 is a highly evolved clone that predominates in human and pig infections in both Asia and Europe.[16] *S. suis* ST1 strains have also been associated with invasive diseases, such as meningitis, in both swine and humans in Thailand and Vietnam.[17,18] More recently, a new sequence type, ST7, was reported as the cause of a Chinese outbreak in 2005.[19] Many patients affected by *S. suis* ST7 strains developed streptococcal toxic shock-like syndrome (STSLS), resulting in a high mortality rate.[19] However, this clinical manifestation was also found in humans infected with ST1 strains in Vietnam,[18] but was rarely reported in patients infected with *S. suis* ST1 strains in Thailand.[20] Interestingly, and differently from Europe and Asia, most North American strains (including those isolated from human patients) belong to either ST25 or ST28.[21] However, ST25 and ST28 were frequently found in human patients from Thailand. In addition, new STs associated with human infections have been recently reported as ST104 and ST20 in human patients from Thailand and the Netherlands, respectively.[16,17] The data of ST *S. suis* infected human patients from 2002 to 2013 are summarized in **Table 3.2**.

The most common STs for *SS2* isolated from diseased pigs during 2002 to 2013 in Canada were ST25 (54%) and ST28 (46%). For United States, the highest prevalence ST was ST28 (75%) followed by ST25 (10%) and ST1 (5%), respectively. Whereas, the ST1 was predominated in *SS2* isolates from diseased pigs in the Netherland, Spain and United Kingdom. In Asia, only China and Japan reported on the STs of *SS2* isolates collected from diseased pigs. In China, ST7 was the most common (77%) followed by ST1 (22%). However, ST28 (76%) was the most common and associated with endocarditis cases in pigs.

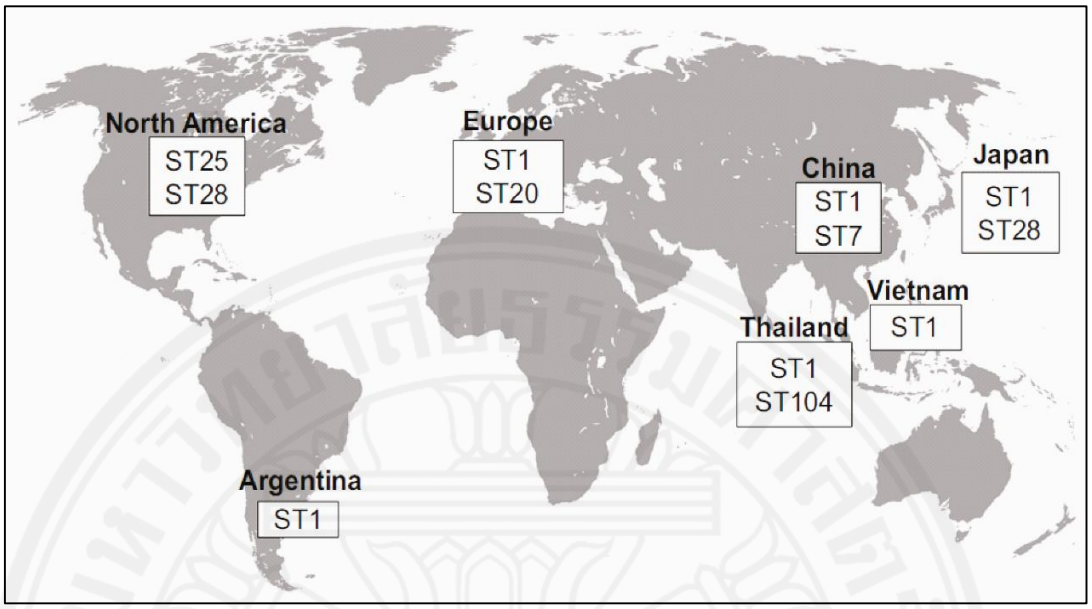


Figure 3.5 Worldwide distribution of the most ST(s) of *SS2* isolates from diseased pigs and human patients during 2002 to 2013[57]

Table 3.2 Sequence types of *S. suis* infected human patients from 2002 to 2013

Country	Serotype	ST	ST complex	Number of cases
North America				
Canada	2	25	25	3
	14	6	1	1
United States	2	1	1	1
		25	25	1
South America				
Argentina	2	1	1	1
French Guiana	2	1	1	1
Europe				
France	2	20	147	2
Italy	2	1	1	1
		134	1	2
Netherland	2	1	1	14
		20	147	11
		134	1	1
		146	1	1
	14	6	1	1
Spain	2	3	1	1
United Kingdom	2	1	1	1
	14	2	1	1
Asia				
Cambodia	2	1	1	13
Mainland China	2	1	1	11
		7	7	210
	14	1	1	1
Hong Kong	2	1	1	14
		9	1	12
		25	25	1
Japan	2	1	1	7
		28	28	1
Thailand	2	1	1	123
		25	25	17
		101	225	1
		102	25	2
		103	25	6
		104	225	45
		126	1	3
	5	181	Unrelated	1
	14	11	11	1
		105	1	19
		127	1	1
	24	221	221/234	1
Vietnam	2	1	1	56
	14	105	1	1
	16	106	Unrelated	1
		101	225	1
		102	25	2
		103	25	6
		104	225	45
		126	1	3
	5	181	Unrelated	1

3.7.2 RAPD-PCR

RAPD-PCR is one of the most simply method for producing the fingerprint for study of intraspecies genetic relatedness. This technique, unlike variable number of tandem repeat (VNTR) PCR, does not require whole genome DNA sequencing but using a single oligonucleotide primer that has partial similarity in DNA sequence with the target DNA. A primer can anneal many target sites of genome and produce PCR variable size of PCR products of many sizes. If the microorganisms are closely genetical related, they provide the same amplicon profile after agarose gel electrophoresis. In case of having a variation on RAPD-primer target sequences, they provide different pattern of DNA banding.[127]

Principle of RAPD-PCR is using the oligonucleotide primer (8–10 bases long) to amplify the respective sequence from genomic DNA under low stringency annealing temperature by PCR. At the satisfied annealing temperature during the amplification, this primer binds randomly with the multiple priming sites in the genomic DNA template and produces PCR product with various sizes. The discrete DNA banding patterns of PCR products could be visualized following agarose gel electrophoresis (**Figure 3.6A**).[127,128] As a consequence amplicon from the same allele that differs in size and will be either presence or absence in the visualized DNA banding patterns.

Chatellier and colleagues[13] have studied the genetic diversity of 88 *SS2* isolates which were isolated from diseased pigs and humans in Europe and North America by using RAPD-PCR analysis with primers OPB7, OPB10 and OPB17. For all three primers, 12 RAPD patterns were conducted with OPB7, 14 RAPD patterns were observed with OPB10 and 11 RAPD patterns were resulted from OPB 17. However, the most 4 RAPD patterns per each primer were reproducibly achieved as shown in **Figure 3.6B**. A cluster analysis based on all RAPD patterns revealed that the diversity of *SS2* isolates from North America and Europe possibly originate from a common ancestor, and all isolates possessing an $MRP^{+}EF^{+}SLY^{+}$ phenotype were found to cluster in a single RAPD fingerprint, regardless of their geographical origin.[13] In addition, only one cluster had RAPD patterns from all *S. suis* carried $MRP^{-}EF^{-}SLY^{-}$

phenotype. This study indicated that RAPD clusters were usually related to EF, MRP and SLY phenotypes, rather than to the geographic or host origins of the *S. suis* strains. Moreover, these three primers gave the reproducible patterns and could successfully assess clonal relationship of *S. suis* strains isolated from infected pigs and humans.

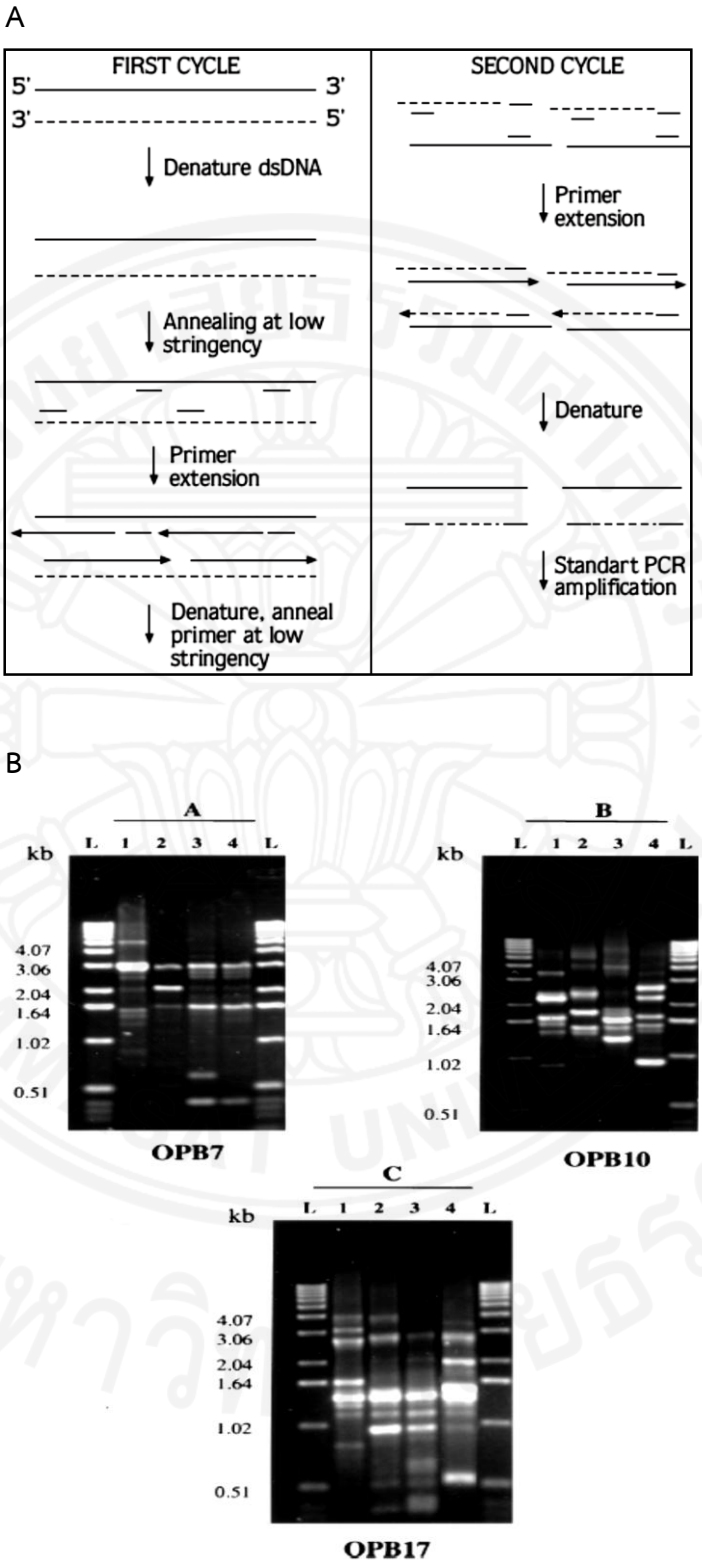


Figure 3.6 RAPD (Panel A) Schematic diagram of RAPD reaction for 2 loci[127,128] and (Panel B) RAPD patterns most frequently generated with primers OPB7, OPB10 and OPB 17[13]

CHAPTER 4
MATERIALS AND METHODS

4.1 *S. suis* isolates

A total of 337 alpha hemolytic streptococci field strains were collected and 245 isolates were identified to be *S. suis* based on conventional biochemical and molecular methods. *S. suis* were isolated from human patients (15 from epidemic and 12 from sporadic cases) and pigs (24 presenting clinical signs of infection and 194 healthy) from three geographical regions of Thailand (**Figure 4.1** and **Table 4.1**). In patients with meningitis, *S. suis* were isolated from both blood and cerebrospinal fluid (CSF), whereas in non-meningitis cases, bacteria were collected from blood only. The 15 human epidemic isolates were obtained from patients during an April–May 2007 outbreak in a Northern endemic region of Thailand (Phayao Province). Among the 12 sporadic case isolates, four were isolated from patients in endemic areas (either the Phrae or Phayao Province) from 2006 to March 2007 (prior to the outbreak) and obtained from the collection of the Ministry of Public Health. The other seven isolates were recovered from sporadic cases after the outbreak had been declared, from the Phayao Province. The remaining isolate was recovered from a patient in the Phayao contiguous Phrae Province. The 24 *S. suis* isolates from diseased pigs were collected from blood. Among these, 12 isolates were from the Phayao Province and six from the Nakhon Pathom and Nakhon Si Thammarat Provinces, respectively. The remaining 194 isolates were obtained from whole tonsil homogenates of slaughterhouse pigs (considered as isolates from healthy pigs) in the Phayao Province during the 2007 epidemic period. In addition, *SS2* strain P1/7 was isolated from an ante-mortem blood culture from a pig dying with meningitis[49] that was used as a reference strain. All bacterial strains have been stored in brain heart infusion (BHI) broth supplemented with 50% glycerol at −80 ºC, at Molecular Microbiology and Immunology Unit, Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University.

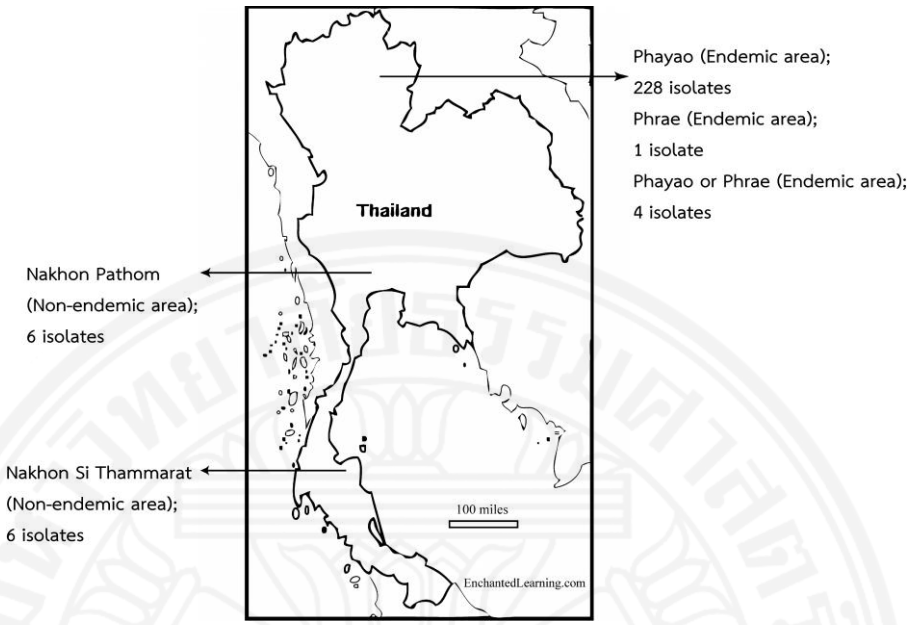


Figure 4.1 Map of Thailand with the locations of the provinces where *S. suis* strains were isolated from humans and pigs[129]

Table 4.1 245 *S.suis* isolates used in this study

Source (Total no. of isolates)	**Province (region) of isolation**	**No. of isolates**	**No. of *SS2* isolates**
Epidemic human cases (15)			
Meningitis (CSF)	Phayao Province (Northern)	8	8
Non-meningitis (Blood)	Phayao Province (Northern)	7	7
Sporadic human cases (12)			
Meningitis (CSF)	Phayao Province (Northern)	4	4
Non-meningitis (Blood)	Ministry of Public Health (Northern)	4	4
	Phayao Province (Northern)		
	Phrae Province (Northern)	3	3
		1	1
Diseased pigs (24)			
Blood	Phayao Province (Northern)	12	0
Blood	Nakhon Pathom Province (Central Plane)	6	2
Blood	Nakhon Si Thammarat Province (Southern)	6	0
Healthy pigs (194)			
Tonsil homogenate	Phayao Province	194	7

4.2 Bacterial strains and plasmids

Escherichia coli (*E. coli*) K-12 and *SS2* strains, and plasmids used in this study are shown in **Table 4.2** and **Table 4.3**.

Table 4.2 Bacterial strains used in this study

Strains	Relevant Genotypes	References
HE06 *SS2*	*SS2* isolated from meningitis patient carrying VAGP1 and ST1	This study
E. coli JM109	F', *traD36 proA*$^{+}$ *B*$^{+}$ *lacq*^{q} *Δ(lacZ) M15/Δ(lac-proAB) glnV44e14*$^{-}$ *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	[130]
E. coli E2096	*E. coli* DH5 harbouring pGP 1-2	[131]
E. coli DH5α	F^{-}, *deoR*, *supE44 Δ(lacZYA-argF) U169 [ø80 dlacΔ(lacZM15)] hsdR17*($r_k^-K^-$) *recA1 endA1 gyrA96 thi-1 relA1,* λ^-	Bethesda Research laboratory, Gaitherburg, MD, USA

Table 4.3 Plasmids used in this study

Vectors	Description	Sources or references
pTA	Cloning vector, Amp^{R}	RBC Bioscience, New Taipei, Taiwan
pET23a+	Expression vector, Amp^{R}	Novagen, Darmstadt, Germany
pVA891	Shuttle suicide vector, Cm^{R}&Erm ^{R}	Paton Laboratory
pGEM-T easy	Cloning vector, Amp^{R}	Promega Biotech, Madison, Wis, USA
pSET4S	Shuttle thermosensitive suicide vector, Spc^{R}	[132]

4.3 Oligonucleotides

The oligonucleotides used in this study are listed in **Table 4.4** and were purchased from three companies *i. e.*, Bio Basic Inc., Toronto, Canada, First BASE Laboratories Sdn Bhd, Selangor, Malaysia and Bioneer, Daejeon, Replublic of Korea.

4.4 Identification of *S. suis* isolates

All 337 field isolates were cultured on 5% blood agar plates (Lab M, Lancashire, UK) (**Appendix A**) and incubated at 37 °C in 5% CO_2 for 16–18 h. Alpha hemolytic colonies were selected and further identified by conventional biochemical tests and carbohydrate utilization as previously described[37,42,133] (**Table 4.5**). *S. suis* isolates were classified into non-ABD Lancefield group as they were unable to grow in the presence of bile esculin and 6.5% NaCl. Moreover, they were Optochin resistant. Next, the *S. suis* were distinguished from the other non-ABD streptococci by their inability to produce acetoin from peptone and acid from mannitol, sorbitol and glycerol. However, they could ferment lactose and hydrolyzed starch. Next, the suspected *S. suis* isolates were further subdivided into three groups depending on trehalose, raffinose and inulin fermentations. The first group, *S. suis* was positive for trehalose, raffinose and inulin fermentation. The second group, probable *S. suis* was positive for trehalose but negative for raffinose and providing variable results for inulin fermentation. Finally, the doubtful group was negative for trehalose, raffinose and inulin fermentation (**Table 4.5**).

Following the conventional identification, the suspected *S. suis* colonies were further confirmed for their species by 16S *rDNA*[134] and *gdh*[43] PCR. The serotype of the confirmed 245 *S. suis* isolates were determined by coagglutination using 35 serotype-specific anti-sera at the reference laboratory for *S. suis* serotyping, Faculty of Veterinary Medicine, University of Montreal, Canada. The presence of the *cps2* gene in isolates belonging to serotype 2 was also determined by PCR as previously described.[3] The *SS2* strain P1/7 was used as a positive control for PCR assays and

Table 4.4 The oligonucleotides used in this study

Oligonucleotides	Sequence (5',-3')	Location/Description	Product length (bp)	References
16S rRNA-F	CAGTATTTACCGGCATGGTAGA TAT	*SS2* P1/7 (AM946016.1) (nt 17,151-17,175)	320	[134]
16S rRNA-R	GTAAGATACCGTCAAGTGAGAA	*SS2* P1/7 (AM946016.1) (complementary nt 17,449-17,470)		[134]
gdh-F	GCAGCGTATTCTGTCAAACG	*SS2* P1/7 (AM946016.1) (nt 239,113-239,132)	690	[43]
gdh-R	CCATGGACAGATAAAGATGG	*SS2* P1/7 (AM946016.1) (complementary nt 239,783-239,802)		[43]
cps2-F	TGATAGTGATTTGTCGGGAGGG	*SS2* P1/7 (AM946016.1) (nt14,572-14,593)	557	[43]
cps2-R	GAGTATCTAAAGAATGCCTATT G	*SS2* P1/7 (AM946016.1) (complementary nt 15,106-15,128)		[43]
epf-F	ATCTACTGGGTATCCTTCTGC	*SS2* P1/7 (AM946016.1) (nt 162,528-162,548)	626	This study
epf-R	CTATCTGGATCTGTGATTGGA	*SS2* P1/7 (AM946016.1) (complementary nt 163,133 -163,153)		This study
mrp-F	TGCTGAAAATACGAGTGC	*SS2* P1/7 (AM946016.1) (nt 732,947-732,964)	953	This study
mrp-R	TGCCADCATAATCATACCC	*SS2* P1/7 (AM946016.1) (complementary nt 733,881-733,899)		This study
sly-F	ACTCTATCACCTCATCCGC	*SS2* P1/7 (AM946016.1) (nt 1,260,077-1,260,095)	1,492	[135]
sly-R	ATGAGAAAAAGTTCGCACTTG	*SS2* P1/7 (AM946016.1) (complementary nt 1,261,548-1,261,568)		[135]
hyl-F	CTCAGATGAAAGCCTTTCTA	*SS2* P1/7 (AM946016.1) (nt 2,412-2,431)	1,295	[106]
hyl-R	TTTGTCCTTGGTCGTTGTC	*SS2* P1/7 (AM946016.1) (complementary nt 3,688-3,706)		[106]

D, a single letter code standing for nucleotide, A or G or T (Not C)

Table 4.4 The oligonucleotides used in this study (Cont.)

Oligonucleotides	Sequence (5',-3')	Location/Description	Product length (bp)	References
arcA-B F	GATGCCTTTGCTCAAGCTCT	*SS2* P1/7 (AM946016.1) (nt 603,928-603,947)	403	This study
arcA-B R	TTTCACGGTTCCGTGTTTCT	*SS2* P1/7 (AM946016.1) (complementary nt 604,311-604,330)		This study
bay046-F	ATGCCACGGATTACCTTCCC	*SS2* P1/7 (AM946016.1) (nt 287,741-287,760)	254	[11]
bay046-R	CCGTCTCCTTAATGATCCGC	*SS2* P1/7 (AM946016.1) (complementary nt 287,975-287,994)		[11]
OPB7	GGTGACGCAG	*SS2* P1/7 (AM946016.1)		[13]
OPB10	CTGCTGGGAC	*SS2* P1/7 (AM946016.1)		[13]
OPB17	AGGGAACGAG	*SS2* P1/7 (AM946016.1)		[13]
aroA-up	TTCCATGTGCTTGAGTCGCTA	*SS2* P1/7 (AM946016.1) (nt 585,356-585,376)	557	[125]
aroA-dn	ACGTGACCTACCTCCGTTGAC	*SS2* P1/7 (AM946016.1) (complementary nt584,820-584,840)		[125]
cpn-up	TTGAAAAACGTRACKGCAGGTG C	*SS2* P1/7 (AM946016.1) (nt 134,580-134,602)	506	[125]
cpn-dn	ACGTTGAAIGTACCACGAATC	*SS2* P1/7 (AM946016.1) (complementary nt 135,065-135,085)		[125]
dpr-up	CGTCTTTCAGCCCGCGTCCA	*SS2* P1/7 (AM946016.1) (nt 1,515,125-1,515,144	462	[125]
dpr-dn	GACCAAGTTCTGCCTGCAGC	*SS2* P1/7 (AM946016.1) (complementary nt 1,514,683-1,514,702)		[125]
gki-up	GGAGCCTATAACCTCAACTGG	*SS2* P1/7 (AM946016.1) (nt 810,819-810,839)	546	[125]
gki-dn	AAGAACGATGTAGGCAGGATT	*SS2* P1/7 (AM946016.1) (complementary nt 811,344-811,364)		[125]

R, a single letter code standing for nucleotide, A or G. K, a single letter code standing for nucleotide, T or G. I, a single letter code standing for inosin

Table 4.4 The oligonucleotides used in this study (Cont.)

Oligonucleotides	Sequence (5',-3')	Location/Description	Product length (bp)	References
mutS-up	AAGCAGGCAGTCGGCGTGGT	*SS2* P1/7 (AM946016.1) (nt1,941,328-1,941,347)	544	[126]
mutS-dn	AGTACAAACTACCATGCTTC	*SS2* P1/7 (AM946016.1) (complementary nt1,940,804-1,940,823)		[125]
recA-up	TATGATGAGTCAGGCCATG	*SS2* P1/7 (AM946016.1) (nt 68,233-68,247)	424	[125]
recA-dn	CGCTTAGCATTTTCAGAACC	*SS2* P1/7 (AM946016.1) (complementary nt 68,639-68,656)		[125]
thrA-up	GATTCAGAACGTCGCTTTGT	*SS2* P1/7 (AM946016.1) (nt 1,618,083-1,618,102)	575	[125]
thrA-dn	AAGTTTTCATAGAGGTCAGC	*SS2* P1/7 (AM946016.1) (complementary nt 1,618,638-1,618,657)		[125]
thrA-forward	AAGAATGGATCATCAACCGT	*SS2* P1/7 (AM946016.1) (nt1,618,210-1,618,229)		[125]
M13F (-40)	GTTTTCCCAGTCACGAC	M13/pUC Forward Sequencing primer (nt 359-375)	170	RBC Bioscience, Taiwan
M13R (-40)	CAGGAAACAGCTATGAC	M13/pUC Reverse Sequencing primer (nt 528-507)		RBC Bioscience, Taiwan
T7 promoter	TAATACGACTCACTATAGGG	pET-23a+ vector (nt 303-319)	294	Novagen, Germany
T7 terminator	GCTAGTTATTGCTCAGCGG	pET-23a+ vector (nt 26-72)		Novagen, Germany
arcA-A F	AAGAATTCATGTCAAACCATCC AATTCATG (*Eco*RI restriction site incorporating is underlined)	*arcA* gene in P1/7 *SS2* (AM946016.1) (nt 603,769-603,790)	1,227	This study
arcA-A R	TTAGCGGCCGCGATGTCTTCAC GTTCAAACGGCA (*Not*I restriction site cooperating is underlined	*arcA* gene in P1/7 *SS2* (AM946016.1) (complementary nt 604,976-604,995)		This study

Table 4.4 The oligonucleotides used in this study (Cont.)

Oligonucleotides	Sequence (5',-3')	Location/Description	Product length (bp)	References
R3 reverse	GGATCCTGAATTTACGGTTGTTAG (*Bam*HI restriction site cooperating is underlined)	*arcA* gene in P1/7 *SS2* (AM946016.1) (nt 604,565-604,582)		This study
F pVA_Cm	CCATGGTGAAAACGGGG	*cat* gene in pVA891 (nt 3,945-3,961)		This study
Forward ups	TGTTAAAATAGATAGAAGGATACT	Upstream of *arcA* gene in P1/7 *SS2* (AM946016.1) (nt 603,745-603,768)	3,227	This study
Reverse dns	AGGATAGCGCCTGTTACCAA	Downstream of *arcA* gene in P1/7 *SS2* (AM946016.1) (nt 604,996-605,015)		This study
FC inverse	ATATTGCTAGCCATGAATTGGATG GTTTGACAT (*Nhe*I restriction site cooperating is underlined)	Inverse PCR primer for amplify *arcA* flanking regions and pSET4S vector sequence		This study
RC inverse	ATATTGCTAGCTGCCGTTTGAACG TGAAGAC(*Nhe*I restriction site cooperating is underlined)	Inverse PCR primer for amplify *arcA* flanking regions and pSET4S vector sequence		This study
F pSET4S	AGATCTCGGTGATGACGGTGAAAA CC	pSET4S (nt 1,662-1,687)	1,715	This study
R pSET4S	ACTAGTTATCTACACGACGGGG	pSET4S (nt 3,355-3,376)		This study
gfp forward	ATATTGCTAGCATGAGTAAAGGAG AAGAACTTTT(*Nhe*I restriction site cooperating is underlined)	*gfp* sequence in pGFPuv (nt 295-317)	750	This study
gfp reverse	ATATTGCTAGCTTATTTGTAGAGC TCATCCATG(*Nhe*I restriction site cooperating is underlined)	*gfp* sequence in pGFPuv (nt 1,023-1,044)		This study

Table 4.5 Conventional biochemical characteristics for *S. suis*

No	Biochemical test	**% positive isolates**[40]			
		Rapid multi-test system[40]			**Microplate identification system**[40]
		SS1	***SS2***	***SS9-SS22***	***SS1-SS8* and *SS1/2***
1	Alpha hemolysis	100	100	100	100
2	Catalase	0	0	0	0
3	Resistance to Optochin susceptibility	100	100	100	100
4	Bile esculin	0	0	0	0
5	6.5% NaCl	0	0	0	0
6	Acetoin (VP)	0	0	0	0
7	Mannitol	1	1	38	4
8	Sorbitol	0	0	4	2
9	Glycerol	0	0	0	ND
10	Lactose	98	100	95	94
11	Starch hydrolysis	100	100	100	87
12	Salicin	100	100	86.5	
13	***Trehalose***	***98***	***99***	***96***	***94***
14	***Raffinose***	***0***	***100***	***88***	***90***
15	Inulin	98	85	75	87

RAPD amplifications, as well as for the MLST analysis.

4.5 Preparation of genomic DNA and DNA quantification

S. suis cells were grown in Todd-Hewitt broth (THB) overnight at 37 ºC. *S. suis* cells were harvested and centrifuged. The bacteria pellets were dissolved in 0.01 M PBS pH 7.4 (**Appendix G**) and centrifuged to wash the pellets. *S. suis* genomic DNA from *S. suis* isolates were then extracted using the Purelink TM genomic DNA

Mini kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Concentration and purity of the DNA were determined by spectrophotometer (Thermo Electron Corporation, USA) at absorbance 260 and 280 nm (A_{260} and A_{280}). For double stranded DNA, a solution containing 50 µg/ml has an A_{260} of 1.0. For purity of DNA, less protein contamination, the ratio of A_{260}:A_{280} should more than 1.2.

4.6 Presence of virulence-associated genes in *S. suis* isolates

Genomic DNA from 245 *S. suis* isolates as described in **Section 4.5** was used as the DNA template for determining the presence of *arcA*, *bay046*, *epf*, *hyl*, *mrp* and *sly* by conventional and multiplex PCR.[136] The sequence of oligonucleotide primers and the respective PCR products are listed in **Table 4.4**. PCRs were composed of 2.5 mM deoxynucleotide triphosphates, 1× PCR buffer (Thermo Scientific, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 10 pmol each of forward and reverse primers (Bio Basic Inc., Toronto, Canada), 1.5 U *Taq* DNA polymerase (Thermo Scientific) and 25 ng of *S. suis* DNA template. The reaction mixtures were completed to 25 µl with ultrapure distilled water. The negative control corresponded to the PCR mixture without the DNA template. The *SS2* strain P1/7 was used as a positive control for conventional and multiplex PCR. The thermocycle programs composed of the initial denaturation at 94 °C for 5 min followed by 35 cycles of amplification (denaturation at 95 °C for 30 sec, annealing at 54 °C for 1 min and extension at 72 °C for 1 min) followed by a final extension of 7 min at 72 °C using a MasterCycler (Eppendorf, Hamburg, Germany). Amplicons were electrophoresed on 1.2% agarose gel (Axygen Biosciences, Union, California, USA) in Tris-Borate-EDTA (TBE) buffer (**Appendix C**) and visualized using an UV transilluminator following ethidium bromide staining (Sigma Chemical Co., St. Louis, MO, USA). A total of 14 virulence-associated genes profiles (VAGPs) were found. The details of 14 VAGPs are summarized in **Table 4.6**.

Table 4.6 Characteristic of VAGPs

VAGP No.	The presence of					
	epf	*mrp*	*sly*	*hyl*	*bay046*	*arcA*
1	+	+	+	+	+	+
2	−	−	+	+	+	+
3	−	−	+	+	−	+
4	+	−	+	+	+	+
5	+	−	−	+	+	+
6	−	+	−	+	+	−
7	−	+	−	+	+	+
8	−	−	+	−	+	+
9	−	−	−	+	−	+
10	−	−	−	+	+	+
11	−	−	−	−	+	+
12	−	−	−	−	+	−
13	−	−	−	−	−	+
14	−	−	−	−	−	−

4.7 DNA fingerprinting by RAPD-PCR

Three RAPD primers, OPB7, OPB10 and OPB17 (**Table 4.4**), were used in this study to amplify *S. suis* genomic DNA as described in **Section 4.5**. The PCR mixtures were prepared as described previously.[13] The cycling program included one cycle of 94 ºC for 4 min, 36 ºC for 1 min and 72 ºC for 2 min; 33 cycles of 94 ºC for 1 min, 36 ºC for 1 min and 72 ºC for 2 min; and a cycle of 94 ºC for 2 min, 36 ºC for 1 min and 72 ºC for 10 min in a MasterCycler (Eppendorf, Hamburg, Germany). Amplification products were analyzed on 1.5% agarose gel in TBE buffer (**Appendix C**) and visualized using an UV transilluminator following ethidium bromide staining (Sigma). The RAPD band patterns visualized by GeneFlash gel documentation system

(Syngene, Cambridge, UK) were further analyzed by band matching software (Syngene, Cambridge, UK). The RAPD band patterns were compared using Dice similarity coefficient and clustered to construct a dendrogram using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) at 3.5% tolerance window (GeneDirectory software, Syngene, Cambridge, UK). The variation between experiments was determined by testing the same *S. suis* strain (including P1/7) in every experiments. The reproducibility of these RAPD patterns was over 99%, and each of the DNA band patterns of the isolates was imported only once into GeneDirectory software.

4.8 Multilocus sequence typing (MLST) analysis

Seven *S. suis* housekeeping genes, a 5-enolpyruvylshikimate 3-phosphate synthase (*aroA*), a 60 kDa chaperonine (*cpn60*), a peroxide resistance protein (*dpr*), a glucose kinase (*gki*), a DNA mismatch repair protein (*mutS*), a homologous recombination factor (*recA*) and an aspartokinase (*thrA*), were amplified for the 36 *SS2* genomic DNA (**Section 4.5**) using the primer sequences listed in **Table 4.4**.[125,126] Each PCR product was purified using either PCR purification kits or agarose gel extraction kits (both from Jena Bioscience GmbH, Jena, Germany). The sequences of both strands of the individual fragments were determined by dye terminator chemistry with primers similar to those used in the initial amplification with the exception of the *thrA* sense primer (**Table 4.4**), on an Applied Biosystems model 3070 automated DNA sequencer. Multilocus sequence typing (MLST) alleles of the individual genes, sequence types (STs) and clonal complexes (CC) were identified using the *S. suis* MLST database and eBURST web application (http://ssuis.mlst.net/).

4.9 Expression and purification of arginine deiminase (ArcA or ADI)

4.9.1 Construction of *E. coli* clone expressing recombinant *SS*2 ArcA

The ArcA-coding DNA sequence was amplified from HE06 *SS2* genomic DNA using specific oligonucleotide primers (*arcA*-A F and *arcA*-A R) (**Table 4.4**). The PCR mixtures were composed of 2.5 mM deoxynucleotide triphosphates, 1x PCR buffer (Thermo Scientific), 2.5 mM $MgCl_2$, 10 pmol forward and reverse primers (Bio Basic), 1.5 U *Taq* DNA polymerase (Thermo Scientific) and 25 ng of *S. suis* genomic DNA. The reaction mixtures were completed to 25 µl with ultrapure distilled water (UDW). The negative control corresponded to the PCR mixture without DNA. The PCRs were achieved using initial denaturation for 4 min, followed by 35 cycles of 30 sec at 94 ºC, 1 min at 54 ºC and 1.5 min at 72 ºC. Final extension was performed at 72 ºC for 7 min. The amplicons were electrophoresed on 1.2% agarose gel (Axygen Biosciences) in TBE buffer and visualized using an UV transilluminator following ethidium bromide staining (Sigma).

4.9.2 Purification of PCR product

PCR products were purified using either PCR purification kits or agarose gel extraction kits (Jena Bioscience GmbH) according to the manufacturer's instruction.

4.9.3 Ligation of ArcA-coding sequence into cloning vector

The purified 1,227-bp *arcA* amplicon containing 3' poly-A overhang was ligated into the T-overhang of the TA vector (**Table 4.3**) using a molar ratio of 3:1 (Insert:Vector) according to the following formula.[137]

$$\text{Insert DNA (ng)} = \frac{\text{vector (ng)} \times \text{insert (kb)} \times \text{(insert/vector ratio)}}{\text{vector (kb)}}$$

The reaction mixture was prepared in total a volume of 20 µl. The mixture was kept at 4 ºC for 16 h and then transformed into JM109 *E. coli*

competent cells (**Section 4.9.4**) by heat shock transformation method (**Section 4.9.5**).

4.9.4 Preparation *E. coli* competent cells using $CaCl_2$-$MgCl_2$ method

E. coli competent cells were prepared according to a method described by Douglas Hanahan.[138] A single *E. coli* colony was inoculated in 5 ml of LB broth at 37 ºC for overnight. Subsequently, one percentage of overnight culture was inoculated in 20 ml of LB broth until the A_{600} reached at 0.4–0.5. The bacterial cells were incubated on ice for 30 min and the supernatants were discarded by using centrifugation at 4,000 *x g*, 4 ºC for 10 min. The pellet was resuspended in 2 ml of pre-chilled 100 mM $MgCl_2$ and centrifuged. The $MgCl_2$-treated cells were then resuspended in 2 ml of cold 100 mM $CaCl_2$ and incubated on ice for at least 60 min, followed by the addition of 450 µl of 80% of glycerol for preservation. These competent cells were divided in 100 µl aliquots and kept at –80 ºC until use.

4.9.5 Heat shock transformation of competent *E. coli* and screening of the transformants

The aliquot of *E. coli* competent cells (**Section 4.9.4**) was removed from –80 ºC and allowed to thaw on ice for 15 min. A ligation mixture (**Section 4.9.3**) was mixed with 100 µl of the *E. coli* competent cells. The reaction was placed on ice for 15–30 min, followed by incubating at 42 ºC for 2 min and placing on ice for 20–30 min. One milliliter of LB broth was added to the mixture and further incubated at 37 ºC for 30 min to allow the bacterial cells to recover and express the antibiotic resistance gene. The transformation mixture was then plated on LB plates supplemented with 100 µg/ml of ampicillin (LB-A) (**Appendix A**), isopropylthio-β-D galactoside (IPTG) and X-gal, and incubated overnight at 37 ºC. Transformant *E. coli* carrying the pTA*::arcA* were identified using blue-white colony screening. The transformant *E. coli* cells containing a *SS2 arcA* inserted into *lacZ* sequence locating in the multiple cloning site (MCS) of pTA would appear as white colonies. They were then screened for the presence of the inserted *arcA* by colony PCR, using *arcA-A* or M13(-40) specific primers (**Table 4.4**).

4.9.6 Preparation of recombinant plasmid DNA

Plasmid DNA was isolated by one of the two following procedures

4.9.6.1 Method 1

Plasmid DNA was subjected to alkaline lysis method.[139] To prepare pTA*::arcA* recombinant plasmid, the *E. coli* carrying pTA*::arcA* was subcultured in 10 ml LB broth supplemented with 100 µg/ml of ampicillin and incubated overnight at 37 ºC. The bacterial cells were harvested by centrifugation. After discarding the supernatant, the cell pellet was resuspended with 300 µl of solution I (**Appendix B**) and incubated for 5 min at room temperature. The mixture was added with 300 µl of solution II (**Appendix B**), followed by keeping on ice for 5 min. Then 300 µl of solution III (**Appendix B**) was added and mixed by vortexing. The bacterial cell debris was removed by centrifugation at 12,000 × *g* for 10 min. The supernatant was transferred into a new tube and then added with an equal volume of chloroform. The upper aqueous phase was collected to a fresh tube after centrifugation at 12,000 × *g*, for 5 min. The upper aqueous phase was added with 0.7 volume of cold isopropanol. The mixture was precipitated at −80 ºC for 30 min or −20 ºC overnight. The precipitated plasmids were sedimented by centrifugation at 12,000 × *g* for 15 min. After discarding the supernatant, the pellet was washed with 1 ml of pre-chilled 70% (V/V) ethanol. The pellet was sedimented 12,000 × *g* for 15 min, dried at 65 ºC for 10 min or under vacuum, and then resuspended in 30−50 µl of ultrapure deionized water (UDW). Integrity of the plasmid was verified by 0.8% agarose gel in TBE buffer and visualized using an UV transilluminator following ethidium bromide staining (Sigma).

4.9.6.2 Method 2

Preparation of plasmid DNA for sequence analysis was performed according to the manufacturer's instruction.

The quality of plasmid was verified by 0.8% agarose gel in TBE buffer and visualized using an UV transilluminator following ethidium bromide staining (Sigma). The amount of plasmid was measured by Nanodrop® spectrometer

(Thermo Electron Corporation, USA) at absorbance 260 and 280 nm as described in **Section 4.5.**

4.9.7 Sub-cloning of ArcA-coding sequence into protein expression vector

Extracted plasmid DNA from **Section 4.9.6.1** was digested with *Eco*RI and *Not*I restriction enzymes, according to the manufacturer's instructions (Thermo Scientific). Briefly, the amount of DNA for cleavage was 0.1–0.5 µg digested with 2–5 U of restriction endonuclease in a final volume of 20 µl. The reaction mixture was incubated at the temperature recommended by manufacturer. For determining quality of the cleavage, the reaction mixture was verified by 0.8% agarose gel electrophoresis. The *Eco*RI/*Not*I-digested *arcA* fragments were purified by using agarose gel extraction kits (Jena Bioscience GmbH). A protein expression vector, pET23a+ (**Table 4.3**), was digested with the same endonucleases and purified by using agarose gel extraction kits.

The purified 1,227-bp *Eco*RI/*Not*I-digested *arcA* (1,227 bp) was further ligated into *Eco*RI/*Not*I-digested pET23a+ vector (3,666 bp) to generate a recombinant plasmid pET23a+*::arcA* (4,893 bp). The recombinant plasmid pET23a+/*arc*A was subsequently transformed into the competent E2096 *E. coli* strain DH5α by heat-shock method at 30 ºC for 5 min and last incubation at 28 ºC for 2 h (**Table 4.2**, **Sections 4.9.4** and **4.9.5**). The mixture was plated on LB selective agar plate containing 100 µg/ml ampicillin and 25 µg/ml kanamycin (LB-AK) (**Appendix A**) and incubated at 28 ºC for 48 h. Colonies of transformed *E. coli* were randomly picked and a LB-AK replica plate was made. Screening of the transformed *E. coli* clones for the presence of the inserted *arcA* gene was performed by direct colony PCR using *arcA-A* or T7 polymerase-specific primers (**Table 4.4**). The *E. coli* clones carrying 1,227 bp *Eco*RI/*Not*I-digested *arcA* were stored as bacterial stocks in LB broth supplemented with 30% sterile glycerol at –80 ºC for further use.

4.9.8 Verification of *arcA*-carrying recombinant plasmid

Recombinant plasmid pET23a+*::arcA* was extracted by method as described in **Section 4.9.6.2** and subjected for DNA sequencing. The correct in-frame fusion of *arcA* fragment in pET23a+*::arcA* was confirmed by DNA sequencing

analysis (FirstBase Laboratories). The raw DNA sequence data were analyzed by the DNAman program (LynnonBiosoft®). Subsequently, the nucleotide sequences and deduced amino acid sequences were analyzed by using Basic Local Alignment Search Tool (BLAST) Program (**Section 4.9.9**).

4.9.9 Analysis of amino acid sequence alignment

The trimmed *arc*A sequence obtained from the pET23a+*::arcA* DNA sequencing analysis was further compared to the other *arcA* homologue existing in the non-redundant nucleotide database using NCBI-BLASTN at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The deduced r*SS2 arc*A amino acid sequence obtained in this study was compared to other ArcA orthologous in the NCBI non-redundant protein database using NCBI-BLASTP. The multiple amino acid alignment of the r*SS2* ArcA and their orthologous proteins amino acid sequence was carried out using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

4.9.10 Expression of r*SS2* ArcA

A single colony of E2096 *E. coli* strain DH5α carrying plasmids pGP1-2 and pET23a+*::arcA* (**Section 4.9.8**) was inoculated into LB broth containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin. The culture was grown at 28 ºC, 250 rpm, overnight. Subsequently, one percent of overnight culture was inoculated into 350 ml of fresh LB broth supplemented with 100 µg/ml of ampicillin and 25 µg/ml of kanamycin and incubated further at 28 ºC with shaking at 250 rpm until the OD_{600nm} reached 0.5. The recombinant protein expression was induced by shifting temperature to 42 ºC for 30 min and followed by further incubation for 6 h at 37 ºC. The bacterial cells were harvested by centrifugation at 4,000 *× g,* 4 ºC for 15 min, discarded supernatants and the cell pellets were the pellets were kept at –80 ºC for further purification (**Section 4.7.12**). The expression of r*SS2* ArcA was investigated by SDS-PAGE and/or Western blot analysis (**Sections 4.8.1**, **4.8.2** and **4.8.3**).

4.9.11 Purification of r*SS2* ArcA

The hexahistidine arginine deiminase fusion protein (His_6-ArcA) was purified from the crude lysate by using Ni-NTA agarose affinity chromatography (Invitrogen) under native condition. The bacterial cell pellets were resuspended in 2x

PBS pH 7.4 supplemented with 20% glycerol and 5 mM dithiothreitol (DTT). The r*SS2* ArcA was purified from the crude lysate (**Section 4.9.10**) by using Ni-NTA agarose affinity chromatography (Invitrogen) under native condition. The bacterial pellets were suspended in 2x PBS extraction buffer (0.02 M of $NaH_2PO_4 \cdot 2H_2O/Na_2HPO_4$ buffer, 300 mM NaCl, 20% glycerol and 5 mM of DTT, pH 7.4) (**Appendix D**), at a ratio of 1.6 g of bacterial pellet per 5 ml of 2x PBS pH 7.4. The bacterial suspension was lysed by sonication (amplitude 35%, pulse on 30 sec and pulse off 30 sec, for 30 min). After cell lysis, the homogenate was separated by centrifugation at 12,000 *× g*, 4 °C for 20 min. Clear supernatant of crude lysate was collected and immediately incubated with Ni-NTA™ agarose beads on ice, allowing binding affinity between the r*SS2* ArcA and beads for 3 h. The beads were then packed into a polystyrene column and extensively washed with a washing buffer (2x PBS extraction buffer, pH 8.0). The r*SS2* ArcA was eluted stepwise from the column using the washing buffer containing 0.1, 0.25, 0.5 and 1.5 mM of EDTA, respectively. The eluted fractions were analyzed by SDS-PAGE and Western-blot. The eluted fractions containing purified r*SS2* ArcA were collected and preserved properly at −80 °C until use.

4.9.12 Verification of r*SS2* ArcA

The eluted fractions were analyzed by 12% sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) and staining by Coomassie Brilliant Blue G-250 dye (Bio-Rad laboratories, CA, USA) (**Section 4.10.1**). The presence of eluted r*SS2* ArcA were confirmed by Western blot analysis using murine anti-His_6-tag antibodies (AbD Serotec, Dusseldorf, Germany).

In addition, the r*SS2* ArcA was verified by mass spectrometry. The stained protein band resolved on SDS-PAGE gel was subjected to LC/MS-MS for protein identification using Finnigan LTQ linear ion-trap mass spectrometer (Thermo Electron Corporation, San Jose, CA), equipped with electrospray ionization (ESI) source and operated in a positive-ion mode. (Protein-Ligand Engineering and

Molecular Biology Laboratory, BIOTEC, The National Science and Technology Development Agency (NSTDA), Thailand).

4.9.13 Production of mouse anti-r*SS2* ArcA polyclonal antibody

All animal experiments were conducted according to the approval from Thammasat University Animal Care and Use Committee (Code. no. 3/2552) (**Appendix F**). Three inbred BALB/C female mice (4 to 6 weeks old) were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. All mice were allowed to adapt to the domesticated condition for three days before commencement the experiment. Mice were immunized with one dose of 5 µg of purified r*SS2* ArcA in Imject® Alum adjuvant (Pierce, Thermo Scientific, Finland) and followed by two doses of 10 µg of purified r*SS2* ArcA in the same adjuvant at 10-day intervals. Blood was collected by cardiac puncture two weeks after the third immunization and serum was stored at 4 ºC. The titers of the anti-r*SS2* ArcA antibodies in the mouse sera were determined by indirect enzyme-linked immunosorbent assay (ELISA).

Titer of the anti-r*SS2* ArcA antibodies in the mouse immune sera was determined by indirect ELISA. One milligram per milliliter of purified r*SS2* ArcA prepared in carbonate-bicarbonate coating buffer (**Appendix G**) was used to coating in 96-wells microtiter plate (Corning, NY 14831, USA) and incubated at 37 ºC for overnight (16–18 h). Subsequently, the r*SS2* ArcA-coated wells were washed three times with washing buffer, PBS-T (**Appendix G**), followed by blocking each well with 200 µl of blocking buffer (**Appendix G**) at 37 ºC for 1 h, and washed three times with PBS-T. After washing unbound proteins, serially diluted mouse sera were individually added to r*SS2* ArcA-coated plated and incubated in a humidified chamber at 37 ºC for 1 h. After incubation, the wells were washed four times with PBS-T, followed by incubation with 100 µl of secondary antibody conjugated with horseradish peroxidase (HRP) at the dilution 1:3,000, at 37 ºC for 1 h and then washed four times with PBS-T. The substrate solution was prepared by dissolving 20 mg of 1, 4-*p*-phenylenediamine dihydrochloride (PPD) (Sigma) in 10 ml of substrate buffer (**Appendix G**), then 10 µl of H_2O_2 were adding into the PPD solution. Next, 10 µl of the substrate solution was

added into each well and kept in the darkness at room temperature for 30 min to allow 2, 3-diaminophenazine development from utilize horseradish peroxidase conjugates. The color reaction was stopped by adding 50 µl of 1 N NaOH and the color was then measured in an ELISA reader at OD_{492nm}. The immune sera were kept at –20 ºC until use.

4.10 Protein analysis

4.10.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously by Laemmli (1970).[140] Samples were suspended in SDS sample buffer (**Appendix H**) and heated to 100 ºC for 3–5 min prior to loading. The samples were centrifuged at 12,000 *x g* for 5 min to obtain a clear supernatant prior to load into the wells. Samples (5–10 µl) were loaded on acrylamide gels on a Mighty® Small II SE250 gel apparatus (Hoefer®, Pharmacia Biotech, USA). Proteins were separated by electrophoresis at 10 mA and 20 mA through a stacking gel (4% acrylamide) and separating gel (12 % acrylamide or otherwise specified), respectively. Protein bands were immobilized by 1 h incubation at room temperature with gentle agitation in fixation solution (**Appendix H**) and stained overnight in staining solution (**Appendix H**). Destaining was achieved with 3 min incubation in neutralization buffer (**Appendix H**) followed by a brief rinse in 25% methanol. The gel was then transferred into the stabilizing solution (**Appendix H**). The resolved protein bands were visualized in blue and could be dried at room temperature by sandwiching between cellophane membranes for long term storage.

4.10.2 Western blot analysis

The method used was a modification of that described by Towbin *et al*.[141] Samples were run on 12% polyacrylamide gels or otherwise specified (**Section 4.4.1**) and transferred to nitrocellulose (Bio-Rad laboratories, CA, USA) at 90 V for 1.5 h in transfer buffer (**Appendix H**) using a using Mini Trans-Blot® Electrophoretic Cell (Bio-Rad laboratories, CA, USA). After transfer the nitrocellulose

sheet was blocked by incubating for 1 h in blocking solution (**Appendix H**), followed by incubation in primary antibody solution (murine anti-His_6-tag antibodies (AbD Serotec, Dusseldorf, Germany) or anti-r*SS2* ArcA antiserum) (appropriately diluted in PBS-T), for 1–16 h. The primary antibody solution was removed and the nitrocellulose sheet was washed four times for 5 min in PBS-T with gentle agitation. Alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad laboratories) was added at a dilution of 1:3,000 in PBS-T, and incubated for at least 1 h with gentle agitation at room temperature. The nitrocellulose sheet was then washed four times for 20 min in PBS-T followed by equilibration in 0.15 M Tris HCl pH 9.6 for 5 min. Membranes were developed by incubation in the dark using the 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) substrate (KPL, MD, USA). The colour reaction was stopped by rinsing with tap water for several times and air dried.

4.10.3 Bradford's assay

Protein concentration were quantified by Bradford's method.[142] Bio-Rad protein assay dye reagent concentrate (Bio-Rad laboratories, CA, USA) was used according to the manufacturer's instruction. The concentration of the protein was calculated from a standard curve constructed by plotting the various amount (0.05 to 0.5 mg/ml) of known bovine serum albumin (BSA) against $OD_{595\ nm}$.

4.11 Characterization of *SS2* ArcA

4.11.1 Citrulline standard curve for ArcA activity determination

Activity assay of ArcA was conducted based on end-point colorimetric method. Various amounts of L-citrulline (0.011, 0.023, 0.046, 0.068, 0.091, 0.114, 0.137, 0.16 and 0.183 µmole) (Sigma) were mixed with 0.25 ml of acid mixtures (one volume of 95% H_2SO_4 to three volumes of 85% H_3PO_4) (**Appendix I**) followed by the addition of 0.25 ml of 3% diacetyl monoxime (**Appendix I**). Next, the reaction mixtures were boiled at 100 ºC in water bath for 15 min to allow the formation of color product that was measured at 490 nm. Amounts of L-citrulline were then plot against $OD_{490\ nm}$.

4.11.2 Determination of r*SS2* ArcA and crude native *SS2* ArcA activities

Activity assay of ArcA was conducted based on colorimetric determination of L-citrulline using diacetyl monoxime.[143] The catalysis of ArcA using arginine as a substrate produces L-citrulline which reacts with diacetyl monoxime in acid solution to form a color product yielding a maximum absorbance (λ_{max}) at 490 nm.[143] Briefly, 0.1 ml of purified r*SS2* ArcA (or crude native *SS2* ArcA) was incubated with 0.4 ml of 10 mM L-arginine (Sigma) in 100 mM potassium phosphate buffer (pH 7.2) (**Appendix I**) at 37 ºC for 2 h to allow the L-citrulline production. After the incubation, the reaction was stopped by addition of acid mixture (**Section 4.11.1**). Subsequently, the reaction was carried out as previously described in **Section 4.11.1**. The amount of L-citrulline produced from the catalysis of ArcA was calculated from a standard curve constructed in **Section 4.11.1**. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of one µmol of L-citrulline per min per ml.

4.11.3 Determination of steady-state kinetic parameters of r*SS2* ArcA and crude native *SS2* ArcA

The amount of r*SS2* ArcA and crude native *SS2* ArcA used in the determination of Michaelis-Menten constants (K_m) and maximum velocity (V_{max}) were 0.3 and 0.1 units, respectively. To determine the K_m and V_{max} values, the assay was conducted in the presence of various concentrations of substrate, L-arginine (0–8 mM), and calculated by fitting the data to the Michaelis-Menten equation using the KaleidaGraph software package (Synergy Software).

4.11.4 r*SS2* ArcA and crude native *SS2* ArcA inhibition assay

To determine the half maximal inhibitory concentrations (IC_{50}) of ArcA inhibitor, L-canavanine (Sigma) or L-N^5-(1-Iminoethyl) ornithine hydrochloride (L-NIO) (Sigma), the 0.3 units of r*SS2* ArcA or 0.08 units of crude native *SS2* ArcA was measured in the presence of various concentrations of L-canavanine (0–20 mM) or L-NIO (0–0.5 mM). The r*SS2* ArcA or crude native *SS2* ArcA was incubated with inhibitors at 25 ºC for 15 min, prior to adding the 10 mM of substrate, L-arginine. The

reaction without the inhibitor was conducted under the same assay condition and taken as the maximum ArcA activity (100% activity).

4.11.5 Expression of crude native ArcA from *SS2* in DMEM by using L-arginine as inducer

To induce the expression of crude native *SS2* ArcA, HE06 *SS2* were grown in Dulbecco's Modified Eagle's medium (DMEM) without L-arginine (Sigma) and L-arginine was used as an inducer. Initially, HE06 *SS2* was cultured on 5% blood agar plates (**Appendix A**) (Lab M, Lancashire, UK) and incubated at 37 ºC in 5% CO_2 for 16–18 h. An isolate colony was subcultured into DMEM without L-arginine supplemented with 10% FBS alone or DMEM supplemented with 10% FBS and 10 mM L-arginine and incubated further for 2, 4, 6 and 18 h. At indicated time points, the bacterial cultures were collected and adjusted to be equivalent to OD_{600} equal to 0.5 (*ca*. 5 × 10^8 CFU/ml) using 2x PBS pH 7.4 (**Appendix I**). Whole cell lysate samples were prepared by sonication (**Section 4.9.11**). The lysates were subjected to ArcA enzymatic activity determination (**Section 4.11.3**) and Western blot analysis using mouse anti-r*SS2* ArcA antiserum (**Section 4.10.2**).

4.11.6 Investigation on a role of ArcA in *SS2* survival under acidic environment

HE06 *SS2* were grown on 5% blood agar plates and incubated at 37 ºC in 5% CO_2 for 16–18 h. The isolated colony was sub-cultured in DMEM. An approximately 1 × 10^7 cfu/ml cell suspensions were grown further in DMEM without L-arginine supplemented with 10% FBS and DMEM without L-arginine supplemented with 10% FBS and 10 mM L-arginine at pH 4.0, 6.0 and 7.5, respectively. Inhibitors, 20 mM L-canavanine and 0.5 mM L-NIO, were added into each culture and further incubated at 37 ºC in 5% CO_2 for 2, 4 and 6 h, respectively. At the indicated times, *SS2* were collected and survival of bacteria were determined by plating 10-fold serial dilution of each culture on 5% blood agar plates and incubated at 37 ºC for 24 h. The cfu of each culture was enumerated. The numbers of *SS2* cultured in the absence and presence of inhibitors in the individual pH were compared. Assays were carried out in triplicate and results were expressed as the mean (± standard error)

cfu/ml. The significance of differences in survival was analyzed using the Mann-Whitney *U* test. The null hypothesis was rejected for *p*-value of > 0.05.

4.12 Construction of a HE06 *SS2 arcA* negative mutant

4.12.1 Construction of *arcA* mutant in HE06 *SS2* based on insertion duplication mutagenesis

4.12.1.1 Cloning of 323-bp *Bam*HI fragment into pVA891 shuttle vector

Genomic DNA of HE06 *SS2* was used as a template for amplification of 814-bp *arcA* gene using *arcA*-A F and R3 reverse primers (**Table 4.4**). The 814-bp PCR product was purified using agarose gel extraction kits (Jena Bioscience) (**Section 4.9.2**) and digested with 0.5 U of *Bam*HI. The resultant, 323-bp *Bam*HI fragment was then ligated into *Bam*HI-digested/dephosphorylated pVA891 at a molar ratio of 3:1 (Insert:Vector) similar to that of previously described (**Table 4.3**, **Sections 4.12.1.2** and **4.9.3**). The ligation mixture was then introduced into chemically competent DH5α *E. coli* by heat shock transformation method (**Table 4.2**, **Sections 4.9.4** and **4.9.5**).

4.12.1.2 Dephosphorylation of restriction endonuclease digested vector

To avoid re-circularization of the restriction endonuclease digested vector in cloning experiments, 0.1–0.5 µg restriction endonuclease-digested vector DNA was treated with 1 U of shrimp alkaline phosphatase (Thermo Scientific) for 30 min at 37 ºC. The reaction was then terminated by heat inactivation of the enzymatic activity at 65 ºC for 15 min.

4.12.1.3 Screening of the *E. coli* transformant carrying pVA891*::*323-bp *arcA*

The chloramphenicol and erythromycin resistant transformant containing pVA891*::*323-bp *arcA* were verified by colony PCR, using F pVA_Cm and R3 reverse primers (**Table 4.4**) and restriction enzyme digestion using *Bam*HI. *E. coli* transformant clone carrying 323-bp *arcA* insert was selected for further

study. The recombinant plasmid from clone pVA891::323-bp *arcA* was extracted (**Section 4.9.6.1**) and purified by plasmid extraction kit (**Section 4.9.6.2**). This plasmid was then further electroporated into competent HE06 *SS2* cells (**Section 4.12.1.4**) using electroporation (**Section 4.12.1.5**).

4.12.1.4 Preparation of the competent HE06 *SS2* for electroporation

Electro-competent HE06 *SS2* cells were prepared according to a method described by Smith *et al*.[144] Overnight cultures in Todd-Hewitt broth (THB) (**Appendix A**) were diluted 50-fold for subculturing into 200 ml fresh THB supplemented with 40 mM DL-threonine and grown until OD_{600} reached 0.4. Cells were then harvested by centrifugation at 2,000 × *g* at 4 ºC for 20 min and the cell pellets were washed twice in 20 ml of ice-cold UDW. Cell pellets were then further washed twice in 20 ml of ice-cold 0.3 M sucrose and followed by washing once with 20 ml of ice-cold 0.3 M sucrose supplemented with 15% (v/v) glycerol. These electro-competent cells were then resuspended in 1 ml of ice-cold 0.3 M sucrose supplemented with 15% (v/v) glycerol and the 50 µl-aliquot were either used directly for electrotransformation or stored at −80 ºC.

4.12.1.5 Electroporation of pVA891::323-bp *arcA* into HE06 *SS2*

Purified recombinant plasmid pVA891::323-bp *arcA* (1−5 µg) were added to 50 µl of electro-competent HE06 *SS2* cells and transferred to prechilled sterile Gene Pulser cuvette (inter-electrode distance 0.2 cm; Bio-Rad Laboratories, CA, USA). Electroporation was carried out using the Bio-Rad Gene Pulser apparatus. Pulses were set at 25 µF, 2.5 kV and 200 Ω. This setting resulted in a time constant ranging from 4.6−5.2 msec. Cells were diluted in 1 ml THB supplemented with 0.3 M sucrose and incubated for 2 h at 37 ºC. These cells were further plated onto Todd-Hewitt agar (THA) plates containing 7.5 µg/ml of chloramphenicol and incubated overnight at 37 ºC.

4.12.2 Construction of *arcA* negative mutant of HE06 *SS2* by using shuttle thermosensitive suicide vector based on allelic exchange mutagenesis

4.12.2.1 Construction of pSET4S*::ΔarcA-gfp*

A 3,227-bp of *arcA* open reading frame and its flanking regions was amplified using primers Forward ups and Reverse dns (**Table 4.4**). The amplicons were resolved by electrophoresis on 1% agarose gel (Axygen Biosciences) in TBE buffer (**Appendix C**) and visualized using an UV transilluminator following ethidium bromide staining (Sigma). The 3,227-bp amplicon was purified and further ligated into the T-overhang of the pGEM-T cloning vector (**Table 4.3**, **Sections 4.9.2** and **4.9.3**). This construct was then transformed into competent DH5α *E. coli* cells (**Table 4.2**, **Sections 4.9.4** and **4.9.5**) and selected by plating on LB agar supplemented with 100 µg/ml amplicillin (**Appendix A**). Insertion of 3,227-bp amplicon into pGEM-T cloning vector was screened by colony PCR using primers (Forward ups and Reverse dns) and (*arcA*-B F and *arcA*-B R) for verification of internal *arcA* fragment (**Table 4.4**). The recombinant plasmid from transformant clone was extracted and analyzed by DNA sequencing analysis (**Sections 4.9.6.2** and **4.9.9**). Subsequently, plasmid pGEM-T*::arcA* DNA of this clone was used as a DNA template for inverse PCR.

The amplicons from inverse PCR was then excised from agarose gel (**Section 4.9.2**), digested with *Nhe*I and recircularization. The recircular plasmid containing only 2,000 bp flanking region of *arcA* but the *arcA* open reading frame was deleted (pGEM-T*::ΔarcA*). Subsequently, this construct was then transformed into competent DH5α *E. coli* cells (**Table 4.2**, **Sections 4.9.4** and **4.9.5**) and selected by plating on LB agar supplemented with 100 µg/ml ampicillin (**Appendix A**). Insertion of 2,000-bp amplicon into pGEM-T cloning vector was screened by colony PCR using primers (M13F(-40) and M13R(-40)), (Forward ups and Reverse dns) and (*arcA*-B F and *arcA*-B R) (**Table 4.4**). A transformant carrying the 2,000 bp flanking regions was selected for further studies. The recombinant plasmid from clone pGEM-T*::ΔarcA* was extracted (**Section 4.9.6.1**) and used as a DNA template for amplifing 2,000-bp fragments using *pfu* DNA polymerase. This purified

PCR product (2,000-bp) was subsequently ligated into *Sma*I digested/dephosphorylated pSET4S vector (**Section 4.12.1.2**) at molar ratio of 10:1 (Insert:Vector) (**Section 4.9.3**). The ligation mixture were introduced into the competent DH5α *E. coli* cells by heat-shock at 30 ºC for 5 min and further incubated at 28 ºC for 2 h before plating on LB selective agar plate containing 50 µg/ml of spectinomycin (**Sections 4.9.4** and **4.9.5**, **Appendix A**). The transformants were selected and screened by colony PCR using F pSET4S and R pSET4S primers (**Table 4.4**). The recombinant plasmid (pSET4S *::ΔarcA*) was extracted, purified and digested with *Nhe*I for insertion of *gfp* from pGFPuv which had been amplified by using *gfp* forward and *gfp* reverse primers (**Table 4.4**). Subsequently, pSET4S*::ΔarcA::gfp* were introduced into the competent DH5α *E. coli* cells (**Sections 4.9.4** and **4.9.5**). The spectinomycin-resistant colonies were selected and screened by colony PCR using *gfp* forward and *gfp* reverse primers. The pSET4S*::ΔarcA::gfp* was extracted (**Section 4.9.6.1**), purified (**Section 4.9.6.2**) and further introduced into the HE06 *SS2* cells by electroporation (**Sections 4.12.2.2** and **4.12.2.3**).

4.12.2.2 Preparation of the electro-competent HE06 *SS2* cells for electrotransformation

Electro-competent *SS2* HE06 cells were prepared according to a method described by Takamatsu *et al*.[145] Briefly, overnight cultures of HE06 *SS2* in THB with 2% yeast extract (THY) (**Appendix A**) were diluted 50-fold in 200 ml fresh THY broth containing 40 mM DL-threonine. HE06 *SS2* were grown further until OD_{600} reached 0.3 to 0.5. Cells were incubated at 50 ºC for 9 min and then harvested by centrifugation at 2,000 × *g* at 4 ºC for 20 min. The pellets were further washed with chemical transformation buffer (CTB) (**Appendix K**) and resuspended in CTB followed by keeping on ice for 30 min. Cells were then washed twice with electroporation buffer (EB) (**Appendix K**) and resuspended in 1 ml of EB containing 15% glycerol.

4.12.2.3 Electroporation of pSET4S*::ΔarcA::gfp* into HE06 *SS2* cells

Purified recombinant plasmid pSET4S*::ΔarcA::gfp* (1–5 µg) was added to 100 µl-aliquot of electro-competent HE06 *SS2* and transferred to the

prechilled sterile electroporation cuvette (2 mm electrode gap, Bio-Rad Laboratories) and pulsed immediately with a Bio-Rad Gene Pulser (2.5 kV, 200 W and 25 mF). The mixture was then diluted immediately with THY broth containing 10% sucrose and 10 mM $MgCl_2$ and further incubated at 28 ºC for 4 h or until reached early log phase. The transformants were divided into two portions.

The first portion was directly spread on THY agar containing 250 µg/ml of spectinomycin and incubated at 28 ºC under 5% CO_2 for 48 h (selection for HE06 *SS2* carrying plasmid pSET4S*::ΔarcA::gfp* and pSET4S*::ΔarcA::gfp* cointegrants). Thereafter, the colonies were selected randomly and subcultured into both blood agar plates and THY broths containing 250 µg/ml of spectinomycin. Blood agar plates incubated at 37 ºC under 5% CO_2 for 24 h (selecting for HE06 *SS2 arcA* negative mutant). The THY broths containing 250 µg/ml of spectinomycin were incubated at 28 ºC until reached early log phase and then shifting temperature at 37 ºC for 4 h followed by spreading on THY agar plates containing 250 µg/ml of spectinomycin and incubated at 28 ºC under 5% CO_2 for 48 h (selecting for HE06 *SS2 arcA* negative mutant).

The second portion was directly shifting temperature for incubation to 37 ºC for 10 h and followed by spreading the transformants on THY agar plates and incubated at 37 ºC under 5% CO_2 for 24 h (selecting for HE06 *SS2 arcA* negative mutant).

CHAPTER 5
RESULTS

5.1 *S. suis* identification

The alpha hemolytic colonies obtained on blood agar plate cultures of each clinical human and pig samples (blood and/or cerebrospinal fluid) as well as whole tonsil homogenates obtained from healthy pigs were subjected to conventional identification based on biochemical testing and carbohydrate utilization (**Section 4.4** and **Table 5.1**). Based on their susceptibility to 6.5% NaCl and bile, resistance to optochin and carbohydrate utilization, 245 of 337 isolates could be classified into the presumptive *S. suis*. Among these, based on trehalose and raffinose utilization, it could be further subdivided into three groups *i. e.,* (i) *S. suis* (positive for trehalose and raffinose utilization tests; 175 isolates), probable *S. suis* (positive for trehalose but negative for raffinose utilization tests; 37 isolates) and (iii) doubtful *S. suis* (negative for trehalose and raffinose utilization tests; 33 isolates).[36,37] (**Table 5.1** and **Table 4.5**). The 337 isolates were further subjected for *S. suis* identification by using *S. suis* specie-specific PCR targeting 16S *rDNA* and *gdh* as described in **Section 4.4.** The *gdh* PCR results confirmed that all 212 isolates belonged to *S. suis* and probable *S. suis* groups based on conventional identification regardless of their origin of isolation were *S. suis*. Whereas only 33 of 125 isolates belonging to doubtful *S. suis* group (26%) were identified to be *S. suis* based on *gdh* PCR results (**Figure 5.1** and **Table 5.1**). These data indicated that the conventional characteristics of *S. suis* isolated from pigs (diseased and healthy) were more diverge compared to that of human (**Table 5.1**). In addition, *S. suis* identification by using *S. suis* specie-specific PCR targeting 16S *rDNA* had lower sensitivity compared to *gdh* in this study (**Table 5.1**).

Table 5.1 Identification of *S. suis* by using conventional and PCR methods

Conventional identification; no. of isolates	**Source of isolation; no. of isolates**	**No. of PCR positive isolates**		**Species identification**
		16S ***rDNA***	***gdh***	
S. suis; 175	Patient (epidemic and sporadic); 27	27	27	*S. suis*
	Diseased pigs; 14	13	14	*S. suis*
	Healthy pigs; 134	111	134	*S. suis*
Probable *S. suis*; 37	Diseased pig;3	2	3	*S. suis*
	Healthy pigs; 34	22	34	*S. suis*
Doubtful *S. suis*; 125	Diseased pig;7	0	7	*S. suis*
	Healthy pigs; 118	22	26	*S. suis*

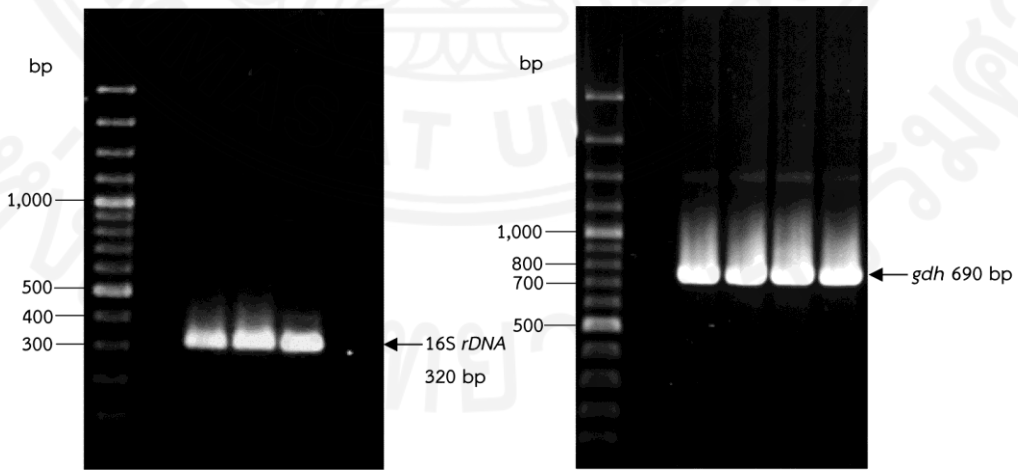


Figure 5.1 PCR amplicons of the 16S *rDNA* and *gdh* from *S. suis* isolates. The target genes are indicated by arrows on the right of the figure. Numbers on the left are the mobility of DNA size markers (GeneRuler^{TM} 100 bp Plus DNA Ladder; Thermo Scientific) in base pairs (bp)

Serotypes of the 245 *S. suis* isolates were performed by coagglutination test using serotype-specific anti-sera to all 35 serotypes.[146] The presence of the *cps2* gene was also determined by PCR[3] (**Section 4.4**). A total of 36 *SS2* isolates were identified by serotyping and *cps2* PCR (**Table 5.2**). All 27 patient isolates were *SS2*, however, *SS2* was not isolated from diseased pigs in the zoonotic endemic Northern Province (Phayao) and the non-endemic Southern Province (Nakhon Si Thammarat), but it was found in two of the six isolates from the non-endemic Central Plain Province (Nakhon Pathom) (**Table 5.2**). On the other hand, seven SS2 isolates were identified among the 194 *S. suis* isolated from healthy pigs in the *S. suis* zoonotic endemic Phayao Province (**Table 5.2**).

For the 209 non-*SS2* isolates, 75 were belonged to 20 different serotypes. Among these, serotype 22 was the most frequently found (20 isolates, 9.6%) followed by serotype 9 (7 isolates, 3.4%), serotypes 3, 16 and 30 (6 isolates each, 2.9%), serotypes 29 and 34 (4 isolates each, 1.9%) and serotype 5 (3 isolates, 1.4%), respectively (**Table 5.2**). The remaining 134 isolates, 94 isolates (45%) were auto-agglutination and 40 isolates (19.1%) were non-typable as they could not provide agglutination to all tested serotype-specific anti-sera. However, these isolates were not belonged to the serotype 2 as they were negative for *cps2*-PCR (**Table 5.2**).

5.2 Virulence genes and genetic diversity of *SS2* Thai isolates

5.2.1 Virulence genes of *SS2* Thai isolates

The presence of six tested virulence-associated genes (*i. e.*, *epf* *mrp*, *sly*, *hyl*, *arcA* and *bay046*) of *SS2* isolates were carried out by PCR (**Section 4.6**). The virulence genes amplification products are shown in **Figure 5.2**. Four virulence-associated gene patterns (VAGP1 to VAGP4) (**Table 4.6**) were found among the isolates (**Table 5.3**). Most of *SS2* isolates (32/36) collected from epidemic and sporadic human cases in Phayao Province and other Northern Provinces, diseased pig in Nakhon Pathom Province (one isolate; non- endemic area) and healthy pigs in Phayao Province (five isolates; endemic area) possessed all tested virulence genes

Table 5.2 Serotypes of 245 *S. suis* isolates in this study

Places of isolation; no. of isolates	**Sources of isolation; no. of isolates**	**Coagglutination**		No. of *cps2* positive isolates
		Serotype	No. of isolate(s) (% of total isolates)	
Phayao Province; 15	***Human (epidemic)***			
	CSF; 4	2	4 (1.63%)	4
	Blood; 11	2	11 (4.49%)	11
†Northern region; 4	Blood; 4	2	4 (1.63%)	4
Phayao Province; 7	***Human (sporadic)***			
	CSF; 2	2	2 (0.82%)	2
	Blood; 5	2	5 (2.04%)	5
Phrae Province; 1	Blood; 1	2	1 (0.41%)	1
Nakhon Pathom Province; 2	***Diseased pigs***			
	Blood; 2	2	2 (0.82%)	2
Phayao Province; 7	***Healthy pigs***			
	Tonsil; 7	2	7 (2.86%)	7
Nakhon Pathom Province; 4	***Diseased pigs***			
	Blood; 4	14	1 (0.41%)	0
		16	1 (0.41%)	0
		22	2 (0.82%)	0
Nakhon Si Thammarat Province; 6	***Diseased pigs***			
	Blood; 6	1	1 (0.41%)	0
		23	1 (0.41%)	0
		25	1 (0.41%)	0
		NT	3 (1.22%)	0

†Ministry of Public Health collection

Table 5.2 Serotypes of 245 *S. suis* isolates in this study (Cont.)

Places of isolation; no. of isolates	**Sources of isolation; no. of isolates**	**Coagglutination**		No. of *cps2* positive isolates
		Serotype	No. of isolate(s) (% of total isolates)	
Phayao Province; 12	***Diseased pigs***			
	Blood; 12	3	1 (0.41%)	0
		34	1 (0.41%)	0
		AA	3 (1.22%)	0
		NT	7 (2.86%)	0
Phayao Province; 187	***Healthy pigs***			
	Tonsil; 187	1	1 (0.41%)	0
		3	5 (2.04%)	0
		5	3 (1.22%)	0
		7	2 (0.82%)	0
		9	7 (2.86%)	0
		12	2 (0.82%)	0
		15	1 (0.41%)	0
		16	5 (2.04%)	0
		19	2 (0.82%)	0
		21	1 (0.41%)	0
		22	18 (7.35%)	0
		24	2 (0.82%)	0
		25	1 (0.41%)	0
		27	1 (0.41%)	0
		28	2 (0.82%)	0
		29	4 (1.63%)	0
		30	6 (2.45%)	0
		34	3 (1.22%)	0
		AA	91 (37.14%)	0
		NT	30 (12.24%)	0

(designated VAGP1). For the remaining four isolates, two isolates each were derived from a sporadic patient in endemic Phayao Province and diseased pig in non-endemic Nakhon Pathom Province lacked both of *epf* and *mrp* genes (designated VAGP2). Interestingly, other two isolates from healthy pigs in Phayao Province, one isolate lacked *epf*, *mrp* and *bay046* gene (designated VAGP3) and another lacked *mrp* only (designated VAGP4).

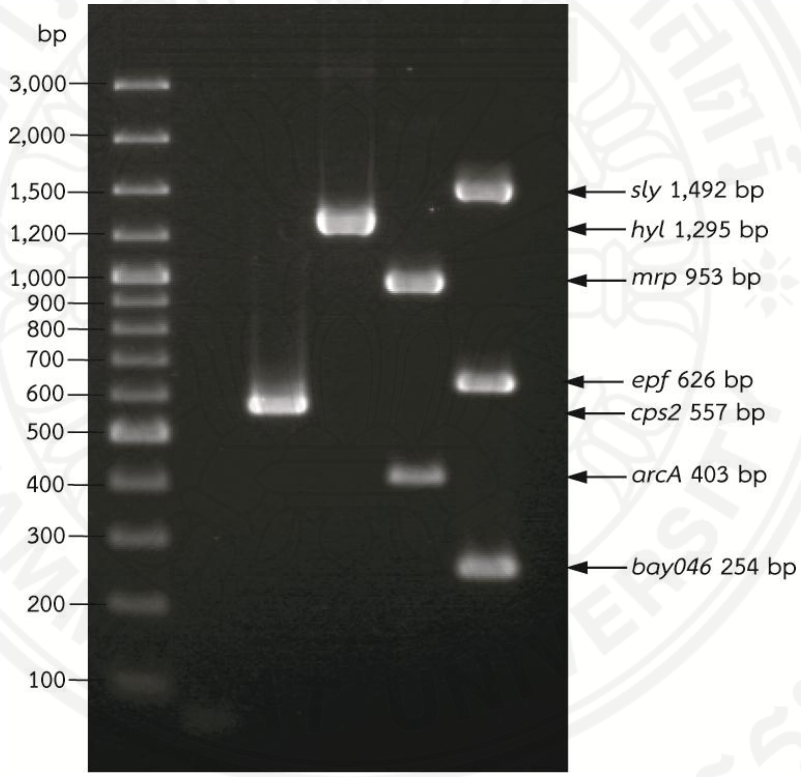


Figure 5.2 PCR amplicons of the *S. suis* virulence-associated genes. The target genes of the PCR amplification products of one isolate from an epidemic human patient are indicated on the right side. Numbers on the left are the mobility of DNA size markers in bp

Table 5.3 Geographical origins, sources of isolation, and characteristics of 36 *SS2* isolates in this study

Places of isolation; no. of isolates	Sources of isolation; no. of isolates	Presence of virulence-associated genes						VAGP	RAPD pattern using primers			ST/CC
		epf	*mrp*	*sly*	*hyl*	*arc*A	*bay046*		OPB7	OPB10	OPB17	
	Human (epidemic)											
Phayao Province; 15	CSF; 4	+	+	+	+	+	+	1	A	A	A	ST1/1
	Blood; 11	+	+	+	+	+	+	1	A	A	A	ST1/1
†Northern region; 4	Blood; 4	+	+	+	+	+	+	1	A	A	A	ST1/1
	Human (sporadic)											
Phayao Province; 7	CSF; 2	+	+	+	+	+	+	1	A	A	A	ST1/1
	Blood; 4	+	+	+	+	+	+	1	A	A	A	ST1/1
	Blood; 1	–	–	+	+	+	+	2	B	B	B	ST104/225
Phrae Province; 1	Blood; 1	+	+	+	+	+	+	1	A	A	A	ST1/1
	Diseased pigs											
Nakhon Pathom Province, Central; 2	Blood; 1	+	+	+	+	+	+	1	A	A	A	ST1/1
	Blood; 1	–	–	+	+	+	+	2	B	B	B	ST104/225
	Healthy pigs											
Phayao Province; 7	Tonsil; 5	+	+	+	+	+	+	1	A	A	A	ST1/1
	Tonsil; 1	–	–	+	+	+	–	3	C	C	C	ST233/–
	Tonsil; 1	+	–	+	+	+	+	4	D	D	D	ST336/–

VAGP, virulence associated genes profile **(Table 4.4)**; ST, sequence type; CC, clonal complex; †Ministry of Public Health collection

5.2.2 Genetic diversity of *SS2* Thai isolates

5.2.2.1 Genetic diversity of *SS2* using RAPD-PCR

Extracted genomic DNA of 36 *SS2* isolates (**Section 4.5**) was subjected to RAPD amplification using three OPB primers, *i. e*., OPB7, OPB10 and OPB17 (**Section 4.7** and **Table 4.4**). Each of the three primers showed 4 RAPD patterns (A to D) with differences in banding number and size. Patterns of up to four bands with sizes ranging between 300 and 3,000 bp were obtained using OPB7. The OPB10 primer produced RAPD patterns with seven to nine bands with a similar size range as those obtained with OPB7. However, OPB17 generated patterns of eight to ten bands with sizes ranging between 100 and 2,000 bp. The RAPD patterns based on the three OPB primers used are shown in **Figures 5.3** and **Table 5.3**. The reproducibility between experiments was determined by testing the P1/7 *S. suis* reference strain in every experiments and the similarity of these RAPD patterns was over 99%. Moreover, a high correlation of RAPD patterns among the three primers used was observed. For example, an isolate with RAPD-A pattern of OPB7 primer also showed the same RAPD-A pattern for OPB10 and OPB17 primers as revealed in **Table 5.3**.

UPGMA-based dendogram construction of the RAPD-A to D patterns from the individual of the three primers used revealed that RAPD patterns from primers OPB7 and OPB17 could categorize the strains of *S. suis* into two unrelated major clusters (Clusters 1 and 2) with some minor differences (**Figures 5.3A** and **5.3C**), whilst, a single major cluster was evident for primer OPB10 (**Figure 5.3B**). Interestingly, these four RAPD patterns were completely correlated to the individual of four virulence gene profiles (**Table 5.2**). Consequently, based on all primers used, all 32 *SS2* isolates belonging to virulence gene profile 1 (VAGP1), regardless of their origins (human or swine), exclusively showed RAPD-A pattern and located exclusively on the cluster 1 of the dendograms constructed from all RAPD primers used (**Figures 5.3**). Two isolates with VAGP2 showed pattern RAPD-B for all primers. Based on dendograms constructed from OPB7 and OPB17 primers, they located on cluster 2 with low similarity to RAPD-A pattern (**Figures 5.3A** and **5.3C**), while, they were assigned on cluster 1 of OPB10 primer-dendogram with 65% similarity to

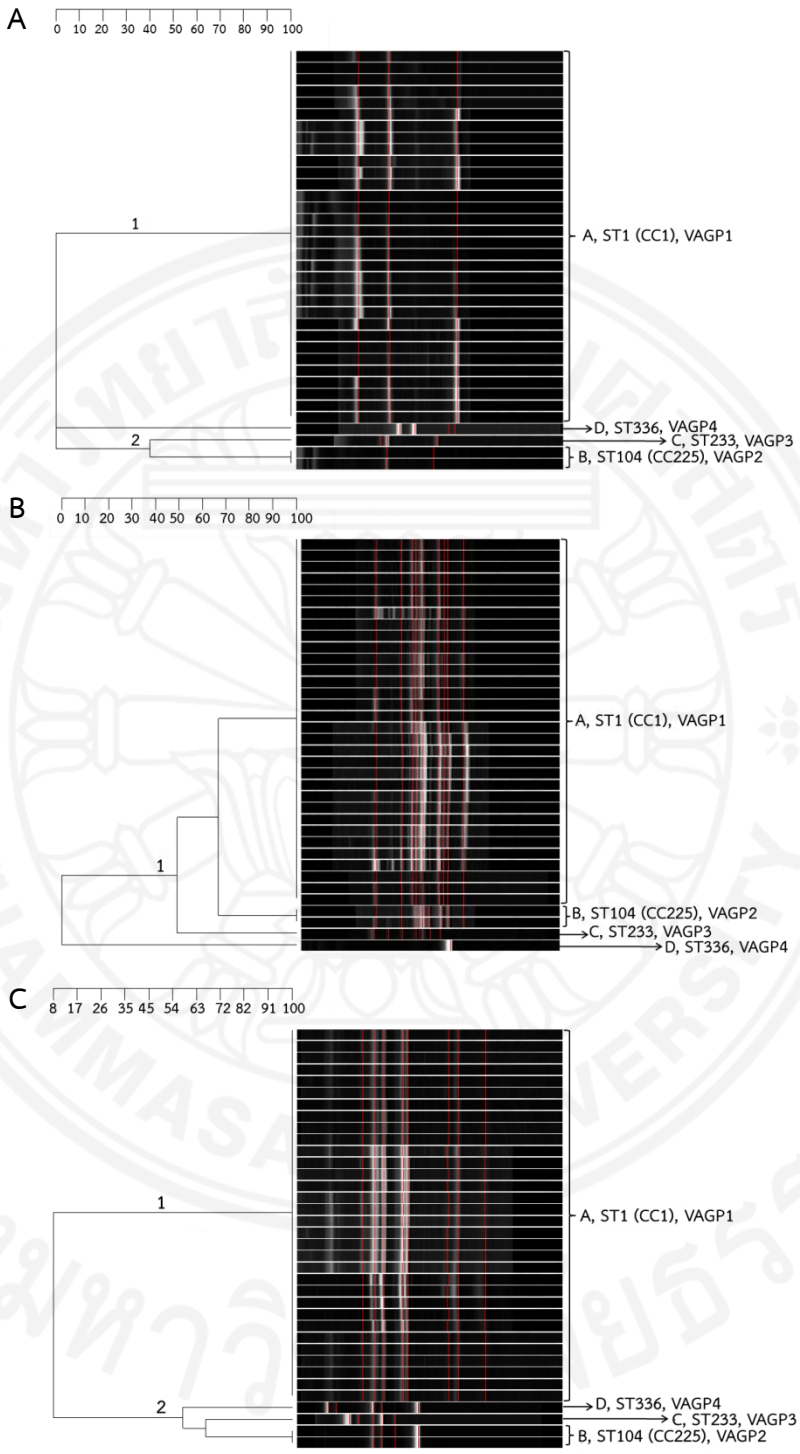


Figure 5.3 Genetic relationship among the 36 isolates of *SS2* as predicted by UPGMA-based clustering analysis of the RAPD patterns generated with primers OPB7 (A), OPB10 (B) and OPB17 (C) and multilocus sequence typing analysis. Each scale on the dendrogram indicates percentage of similarity among RAPD patterns. RAPD patterns sharing 100% similarity were grouped into clusters

RAPD-A (**Figure 5.3B**). A healthy pig isolate in endemic area with VAGP3 showed RAPD-C and was also assigned to cluster 1 of OPB10-based dendogram but it had only 45% similarity to the RAPD-A (**Figure 5.3B**). On the other hands, this isolate was allocated on cluster 2 based on dendograms constructed by OPB7 and OPB17 primers and not related to RAPD-A. However it revealed 40% and 68% similarities for RAPD-B of OPB7 and OPB17, respectively (**Figures 5.3A** and **5.3C**). Another healthy pig isolate in endemic area with VAGP4 showed RAPD-D and was assigned to the separate branch of OPB7- and OPB10-based dendograms with no similarity to the other patterns (**Figures 5.3A** and **5.3B**). Nevertheless, it was assigned to the cluster 2 of OPB17 but it had only 59% similarity to the RAPD-B and C (**Figure 5.3C**).

5.2.2.2 Genetic diversity of *SS2* using multilocus sequence typing (MLST)

MLST analysis using DNA sequencing data of seven conserved housekeeping genes (**Section 4.8** and **Table 4.4**) amplified from genomic DNA of 36 *SS2* isolates revealed four sequence types (STs), *i. e.*, ST1, ST104, ST233 and new ST336 (**Table 5.2**). Interestingly, like the RAPD patterns, the isolates classified into each of the four STs completely conformed to the individual profiles of virulence gene (**Figure 5.3** and **Table 5.2**). Therefore, all 32 *SS2* isolates, that had VAGP1 and RAPD-A, were ST1 and belonged to clonal complex 1 (CC1). Two VAGP2/RAPD-B isolates were assigned into ST104 of CC225. Moreover, an isolate from healthy pigs with VAGP3/RAPD-C was ST233 and it was different from ST104 by the *gki* and *thrA* alleles. The remaining one isolate from healthy pig with VAGP4/RAPD-D was classified to be a new sequence type, ST336 as a result of carrying new alleles for three genes (*cpn60*, *dpr* and *recA*).

5.3 Virulence genes and genetic diversity of non-*SS2* Thai isolates

5.3.1 Virulence genes of non-*SS2* Thai isolates

Thirteen VAGPs (VAGP1–2 and VAGP4–14) (**Table 4.6**) were identified among the 209 non-*SS2* (VAGP3 was absent from non-*SS2* Thai isolates in this study). The VAGPs of the non-*SS2* isolates are summarized in **Table 5.4**. The

majority of non-*SS2* isolates collected from healthy pigs in Phayao Province (92/187 isolates) carried only *arcA* and *bay046* genes (designated as VAGP11). Interestingly, nine of twelve isolates from diseased pigs in endemic Northern Province (Phayao) lacked all tested VAGPs (designated as VAGP14). Remaining of three isolates had *bay046* gene only (designated as VAGP12) (**Table 5.4**). In addition, five of six isolates from diseased pigs in non-endemic zoonotic area (Nakhon Si Thammarat) had VAGP14 (**Table 5.4**). In this study, there were 16 non-*SS2* isolates carrying the same VAGPs to that of the *SS2* isolates (VAGP1–4). Among these, three, seven and six isolates were VAGP1, 2 and 4, respectively.

5.3.2 Genetic diversity of non-*SS2* Thai isolates using RAPD-PCR

Extracted genomic DNA of the 209 non-*SS2* isolates was subjected to RAPD amplification using three OPB primers similar to that of *SS2* isolates (**Section 4.7** and **Table 4.4**). Each of the three primers produced more diverse RAPD patterns than that observed in *SS2* isolates. Up to 12 bands were observed using OPB7. OPB10 primer produced RAPD patterns of 1–14 bands, while OPB17 generated patterns of 1–10 bands. The size ranges of RAPD patterns obtained by all three primers were between 100 and 3,000 bp. However, unlike the *SS2* isolates, no correlation of RAPD patterns among the three primers used was observed in 197 isolates of non-*SS2* isolates. Furthermore, UPGMA-based dendogram construction of the RAPD pattern from the individual of the three primers used revealed that OPB7, OPB10 and OPB17 primers generated dendogram with 121 (181 patterns), 99 (181 patterns) and 100 (181 patterns) branches, respectively. Moreover, no major cluster could be assigned as a consequence of their extremely diverse patterns (**Figures 5.4A** to **5.4C**).

The attention was then focused only on the relationship of RAPD patterns among the 16 non-*SS2* isolates (16 RAPD patterns) carrying VAGP1, VAGP2 and VAGP4 similar to that of *SS2* isolates (**Table 5.4** and **Section 5.3.1**). Interestingly, UPGMA-based dendogram construction of these 20 patterns from the individual of the three primers used revealed that RAPD patterns from OPB7 and OPB17 primers could loosely categorize the non-*SS2* strain into two unrelated major clusters (Clusters 1 and 2) with some differences (**Figures 5.5A** and **5.5C**), whilst, a single

Table 5.4 Geographical origins, sources of isolation and characteristics of 209 non-*SS2* isolates in this study

Places of isolation; no. of isolates	**Sources of isolation; no. of isolates**	**VAGP**	**No. of isolates**
	Diseased pigs		
Nakhon Pathom Province; 4	Blood; 4	1	1
		12	3
Nakhon Si Thammarat Province; 6	Blood; 6	1	1
		14	5
Phayao Province; 12	Blood; 12	12	3
		14	9
Phayao Province; 187	Tonsil; 187	1	1
		2	7
		3	0
		4	6
		5	1
		6	1
		7	1
		8	3
		9	5
		10	1
		11	92
		12	29
		13	13
		14	27

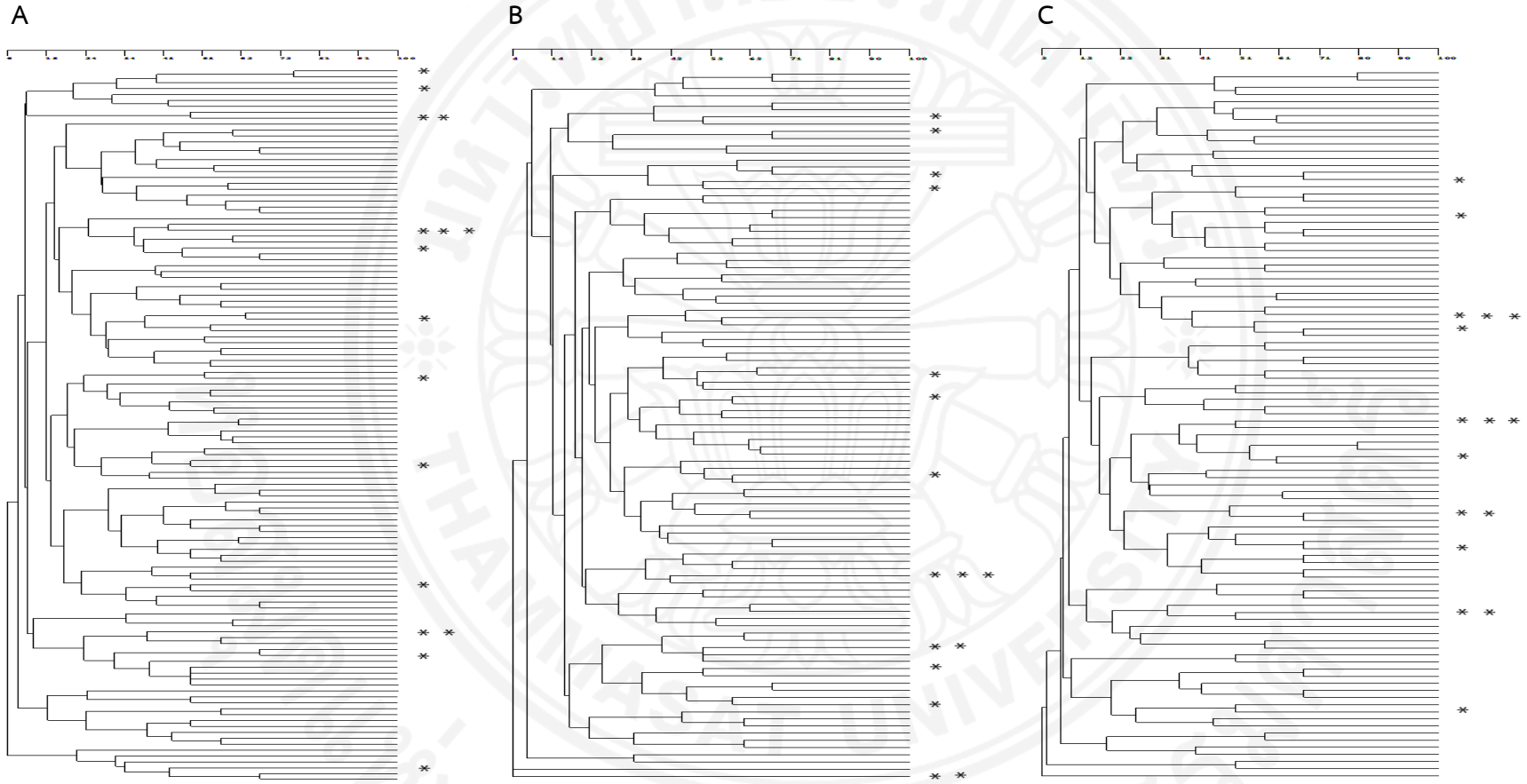


Figure 5.4 The genetic relationship among non-*SS2* that had VAGP1–14 as predicted by UPGMA-based clustering analysis of the RAPD patterns generated with primers OPB7 (A), OPB10 (B) and OPB17 (C). Each scale on the dendrogram indicates percentage of similarity among RAPD patterns. The asterisks indicate 16 non-*SS2* isolates (16 RAPD patterns) that carried the VAGP 1, 2 and 4

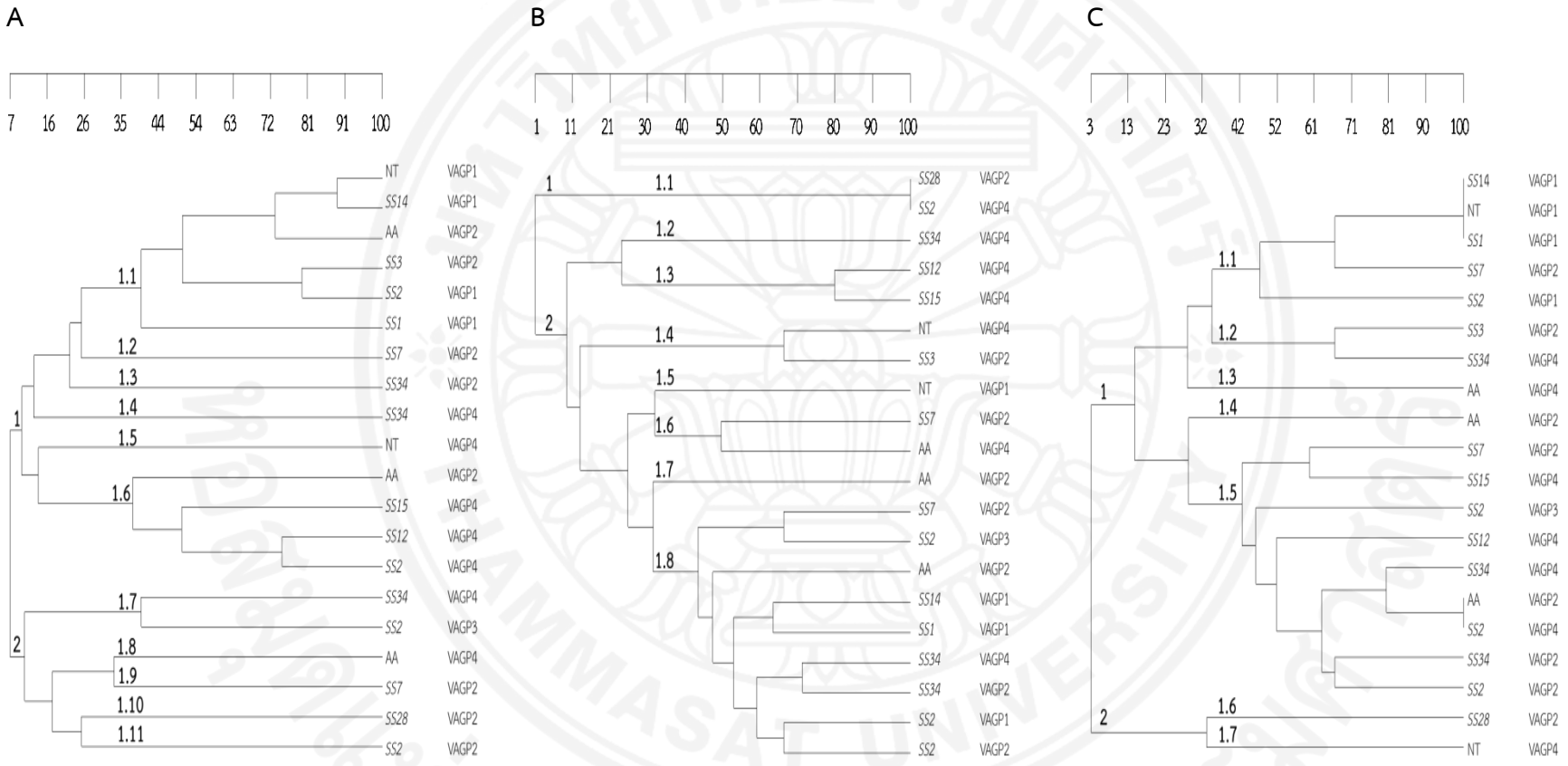


Figure 5.5 Genetic relationship among the 16 isolates of non-*SS2* comparing to *SS2* isolates as predicted by UPGMA-based clustering analysis of the RAPD patterns generated with primers OPB7 (A), OPB10 (B) and OPB17 (C). Each scale on the dendrogram indicates percentage of similarity among RAPD patterns

major cluster was evident for OPB10 primer (**Figure 5.5B**). Although, these RAPD patterns were not completely correlated to the individual of the four virulence gene profiles, geographical origins, and sources of isolation, almost all of these strains were located in the cluster 1 of the dendograms constructed from OPB7 and 17 primers (**Figures 5.5A** and **5.5C**). Moreover, all four non-*SS2* isolates carrying VAGP1 were located exclusively on the branch 1.1 of cluster 1 of the dendograms with over with 35% similarity. While the majority of VAGP2 and 4 isolates were assigned into branches 1.2–1.6 and 1.2–1.5 of the dendograms constructed from OPB7 and 17 primers (**Figures 5.5A** and **5.5C**).

5.4 Characterization of arginine deiminase

Recently, two dimentional electrophoresis followed by immunoblot assay have been employed to identify *in vivo* expressed antigens of *SS2* among three infected species: human, mouse and pig. The obtained data showed that *SS2* arginine deiminase (ArcA) was one of the predominant *in vivo* expressed antigen during infection (Srimanote *et al*., unpublished data and Jing *et al*.[22]). Moreover, previous data in this thesis revealed that all human clinical isolates of *SS2* obtained from Thailand carried *arcA* gene (**Section 5.2.1**). Furthermore, *arcA* appeared to have a high prevalence among non-*SS2* (68.57%) (**Section 5.3.1**). Taken together, the data suggested that the *SS2* ArcA might have a role in either bacterial pathogenesis or persistence in the reservoir.

5.4.1 Construction of recombinant *E. coli* clone expressing *SS2* ArcA

In order to characterize the biological functions of *SS2* ArcA, the construct was designed to link to hexa-histidine tag at the C-terminus of arginine deiminase protein (His_6-ArcA) using pET expression system. The entire *arcA* gene was amplified from genomic DNA of *SS2* strain HE06 (**Section 4.9.1**) using oligonucleotide primers *arcA*-A F and *arcA*-A R which incorporated *Eco*RI and *Not*I restriction sites, respectively (**Table 4.4**). The 1,227-bp *arcA* amplicon (**Figure 5.6**) was purified and

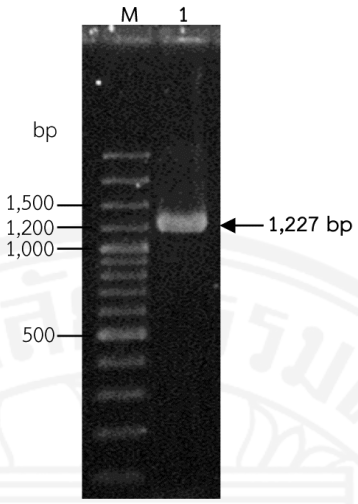


Figure 5.6 PCR amplicon of ArcA-coding sequence
Lane M, GeneRuler^{TM} 100 bp plus DNA ladder
Lane 1, 1,227-bp *SS2 arcA* amplicon (arrow)
Numbers on the left are the mobility of DNA size markers in bp

further ligated into the T-overhang of the TA cloning vector (**Sections 4.9.2** and **4.9.3**, **Table 4.3**) to generate pTA*::arcA*. This construct was then transformed into competent JM109 *E. coli* cells (**Sections 4.9.4** and **4.9.5**) and selected by plating on LB agar supplemented with 100 µg/ml amplicillin (**Appendix A**). Insertion of *arcA* into TA vector (designated pTA*::arcA*) was screened by colony PCR using primers M13F(-40) and M13R(-40) (**Table 4.4**). The PCR product 1,477-bp (including *arcA* and vector multi-cloning sites franking regions) was obtained from 9 out of the 11 randomly selected colonies (**Figure 5.7**). *E. coli* transformant clone number 5 carrying the *SS2 arcA* insert was selected for further study.

The recombinant plasmid pTA*::arcA* was extracted and subjected to DNA sequencing analysis. BLASTN analysis indicated that the nucleotide sequence of *SS2 arcA* derived from strain HE06 was identical to *arcA* sequence of other 10 *SS2* strains in the Genbank database including the reference *SS2* strain P1/7 and China large outbreak strain SC84 as shown in **Figure 5.8**. Subsequently, pTA*::arcA* DNA was digested with *Eco*RI and *Not*I restriction endonuclease enzymes (**Figure 5.9** and

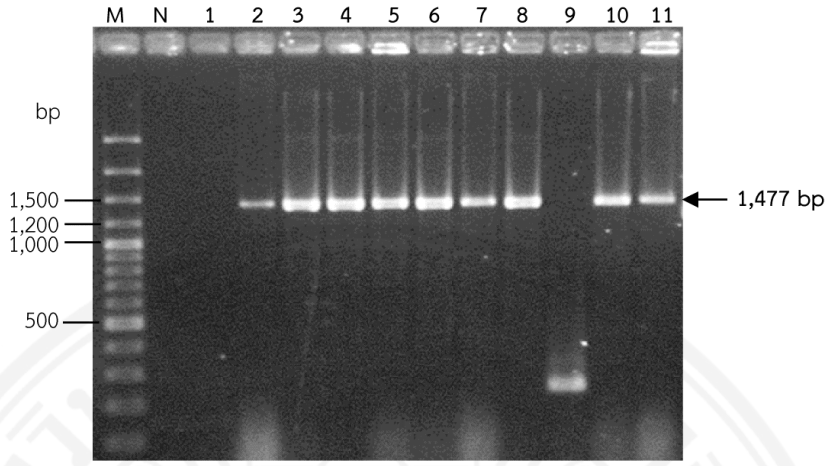


Figure 5.7 *SS2 arcA* PCR amplicons from 11 randomly selected JM109 *E. coli* transformants. The expected size of amplicon is 1,477 bp (arrow)

Lane M, GeneRuler^{TM} 100 bp plus DNA ladder

Lane N, Negative control (no template)

Lanes 1–11,PCR amplicons of transformant clones, number 1–11

Numbers on the left are the mobility of DNA size markers in bp

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus suis strain ZY05719, complete genome	2266	2266	100%	0.0	100%	CP007497.1
Streptococcus suis SC070731, complete genome	2266	2266	100%	0.0	100%	CP003922.1
Streptococcus suis S735, complete genome	2266	2266	100%	0.0	100%	CP003736.1
Streptococcus suis A7, complete genome	2266	2266	100%	0.0	100%	CP002570.1
Streptococcus suis SS12, complete genome	2266	2266	100%	0.0	100%	CP002640.1
Streptococcus suis JS14, complete genome	2266	2266	100%	0.0	100%	CP002465.1
Streptococcus suis GZ1, complete genome	2266	2266	100%	0.0	100%	CP000837.1
Streptococcus suis P1/7 complete genome	2266	2266	100%	0.0	100%	AM946016.1
Streptococcus suis BM407 complet genome, strain BM407	2266	2266	100%	0.0	100%	FM252032.1
Streptococcus suis SC84 complete genome, strain SC84	2266	2266	100%	0.0	100%	FM252031.1
Streptococcus suis 98HAH33, complete genome	2266	2266	100%	0.0	100%	CP000408.1
Streptococcus suis 05ZYH33, complete genome	2266	2266	100%	0.0	100%	CP000407.1

Figure 5.8 *arcA* DNA sequences alignment using BLASTN search (Available from http://blast.ncbi.nlm.nih.gov/Blast.cgi; Accessed on April 6, 2014)

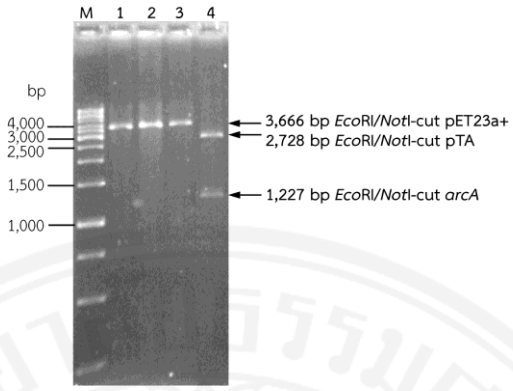


Figure 5.9 *Eco*RI and *Not*I restriction endonuclease digestion of pTA*::arcA* and pET23a+ vector. Following the digestion, the digested DNA mixtures were analyzed by 0.8% TBE-agarose gel electrophoresis

Lane M, GeneRuler^{TM} 1 kb DNA ladder

Lane 1, undigested pET23a+ vector

Lane 2, *Eco*RI/*Not*I digested pET23a+ vector

Lane 3, undigested pTA*::arcA*

Lane 4, *Eco*RI/*Not*I-digested pTA*::arcA*

The *Eco*RI/*Not*I-digested *arcA* (1,227 bp), pTA vector (2,728 bp) and pET23a+ vector (3,666 bp) DNA fragments were indicated by arrows.

Numbers on the left are the mobility of DNA size markers in bp

Section 4.9.7). The 1,227-bp *Eco*RI/*Not*I *arcA* fragment was then excised from agarose gel (**Section 4.9.2**), purified, and ligated into *Eco*RI/*Not*I sites of digested pET23a+ vector and introduced into the competent JM109 *E. coli* cell (**Sections 4.9.4** and **4.9.5**)**.** The ampicillin-resistant colonies were selected and screened by colony PCR using T7 promoter and T7 terminator primers (**Table 4.4**) for the presence of expected 1,502-bp PCR product containing *arcA* flanked by pET23a+ multi-cloning sites (pET23a+ MCS) (**Figure 5.10**). Correct in-frame fusion of the *SS2 arcA* fragment into pET23a+ (designed as pET23a+*::arcA*) was confirmed by DNA sequencing analysis (**Sections 4.9.8** and **4.9.9**). The construction strategy is summarized in **Figure 5.11**. The recombinant plasmid, pET23a+*::arcA* was extracted from clone no. 14 (**Figure 5.10**)

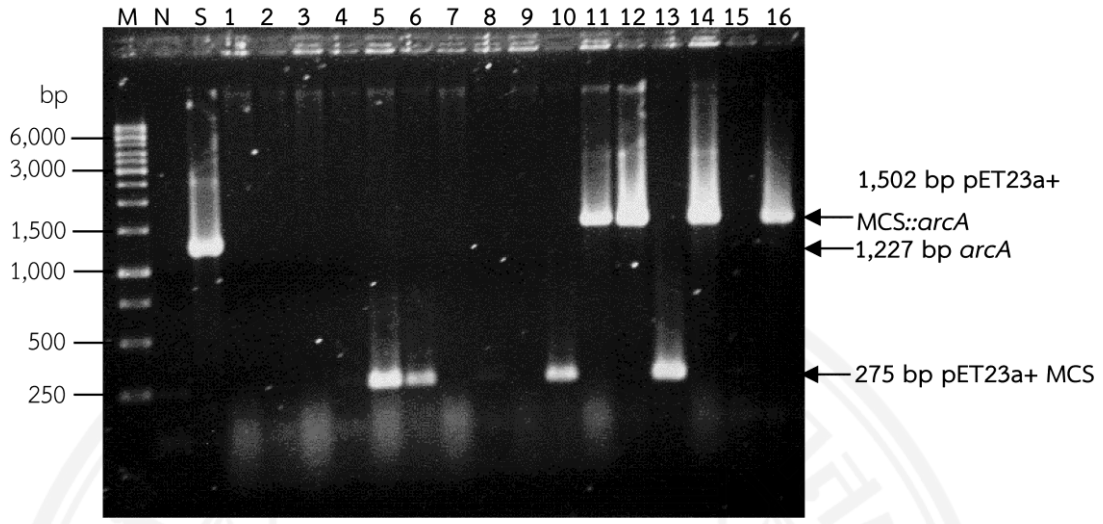


Figure 5.10 *arcA* PCR screening of 16 randomly selected colonies of pET23a+*::arcA* transformed JM109 *E. coli* clones

Lane M, GeneRuler™ 1 kb DNA ladder

Lane N, Negative control (no template)

Lane S, *arcA* amplicon (1,227 bp)

Lane 1–16, PCR amplicon of 16 randomly selected colonies of pET23a+*::arcA* transformed JM109 *E. coli* clones

The expected amplicom sizes of pET23a+ MCS*::arcA* (1,502 bp) and pET23a+ MCS (275 bp) amplicons are indicated by arrows.

Numbers on the left are the mobility of DNA size markers in bp

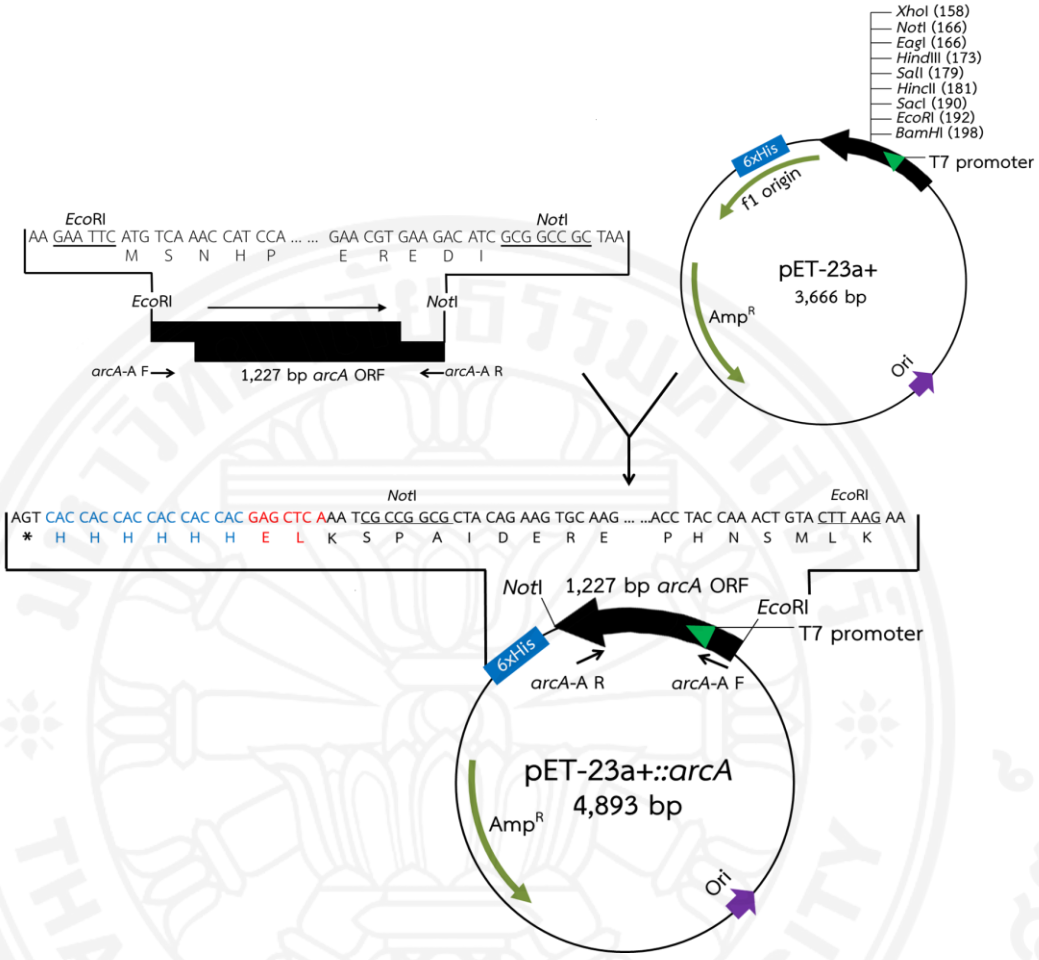


Figure 5.11 Construction for r*SS2* ArcA protein expression. A *SS2 arcA* DNA coding sequence (1,227-bp) was amplified by PCR using oligonucleotides primer *arcA*-A F (*Eco*RI) and *arcA*-A R (*Not*I), digested with *EcoR*I and *Not*I endonuclease and then ligated into the *EcoRI*/*Not*I digested expression vector pET23a+ in order to fuse the C-terminal region of *SS2* ArcA with the His_6-tag to construct plasmid pET23a+*::arcA*. This plasmid was transformed into the JM109 *E. coli.* The plasmid was extracted and transformed into E2096 *E. coli*. The black filled arrow indicates the DNA region of the *SS2 arcA* open reading frame (ORF). The oligonucleotides used are designated by the small arrows accompanied by the primer names. The horizontal long thin arrow indicates the direction of transcription of the *SS2 arcA* ORF in PCR product. The His_6-tag DNA and deduced amino acid sequences are shown in blue. The pET23a+ DNA and deduced amino acid sequences are shown in red. The *SS2* ArcA DNA and deduced amino acid sequences are shown in black. The stop codon is indicated by asterisk. The plasmid maps are not to scale

and transformed into the expression host E2096 *E. coli* (*E. coli* strain DH5 carrying pGP1-2 which contained T7 RNA polymerase gene under the control of temperature sensitive repressor (*cI*857)) (**Table 4.2**). Subsequent to T7-promotor induction by shifting temperature up to 42 ºC (**Section 4.9.10**), *E. coli* whole cell lysate samples were prepared and analyzed by 12% SDS-PAGE and Western blot analysis to visualize r*SS2* ArcA protein band using anti-His_6-tag monoclonal antibodies (**Section 4.10**). The soluble r*SS2* ArcA with a relative molecular mass of approximately 47 kDa (expected size of r*SS2* ArcA) was observed in the induced sample but not in the sample prepared from non-induced E2096 *E. coli* carrying pET23a+*::arcA* (**Figure 5.12**).

5.4.2 r*SS2* ArcA assay

Enzymatic activity of the r*SS2* ArcA was measured by Okinsky's method based on the determination of L-citrulline with maximum absorbance at 490 nm[143] **(Section 4.11.1)**. In order to evaluate enzymatic activity, a standard curve was prepared according to the method as described in **Section 4.11.1**. The absorbance at 490 nm of individual concentrations of L-citrulline were expressed as mean values obtained from triplicate independent experiments as shown in **Table 5.5**. The standard curve was constructed as described in **Section 5.4.5.1**. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of one µmol of L-citrulline per min per ml.

5.4.2.1 Construction of standard curve for r*SS2* ArcA activities

As a consequence of ten A_{490} values obtained from **Table 5.5** did not exhibit the linear relationship, only eight A_{490} values (No. 2–8 in **Table 5.5**) were selected. The linearity was obtained and defined the limit of detection ranged from 0.023 to 0.160 µmole (**Figure 5.13** and **Table 5.5**). In order to allow the increasing of dynamic range of the detection, fitting the standard curve with polynomial equation was used in this study.[147] The non-linear relationship between citrulline and OD_{490} was further used to fit the standard curved. It was found that the non-linear standard curve based on polynomical equation ($y= ax^2+bx-c$) had the limit of detection ranged from 0.006 to 0.183 µmole as shown in **Figure 5.14**. This non-linear standard curve was further used for all experiments in this study.

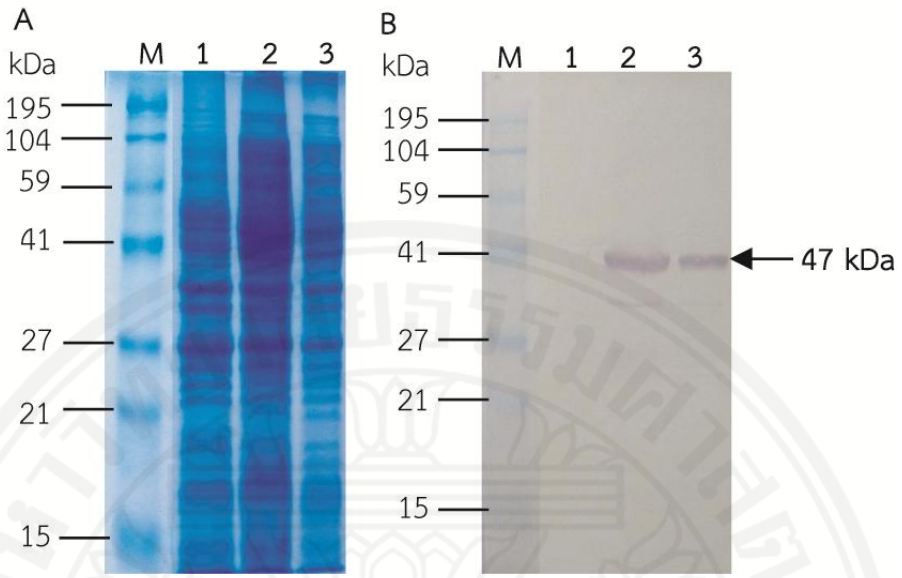


Figure 5.12 r*SS2* ArcA protein expression in E2096 *E. coli*. E2096 *E. coli* carrying pET23a+::*arcA* were induced with temperature shifting. Whole cell samples were collected at 6 h post induction and lysed to prepare soluble (lysate) and insoluble protein (pellet) portions. The samples were then subjected to SDS-PAGE on a 12% gel followed by Western blot analysis. SDS-PAGE of separated samples were stained with Coomassie blue G250 (A) and probed with mouse anti-His_6 tag antibody (B). The approximately 47-kDa of predicted r*SS2* ArcA protein were found in both of soluble and insoluble portions as indicated by arrow

Lane M, Pre-stained broad range protein standard (Bio-Rad Laboratories)

Lane 1, Non-induced sample of E2096 *E. coli* clone carrying pET23a+::*arcA*

Lane 2, Insoluble portion prepared from induced E2096 *E. coli* clone carrying pET23a+::*arcA*

Lane 3, Soluble portion prepared from induced E2096 *E. coli* clone carrying pET23a+::*arcA*

The relative mobility of the expected r*SS2* ArcA with size of 47-kDa is indicated by arrow. Molecular weights of protein markers are indicated as numbers shown on the left of figure

Table 5.5 Absorbance at 490 nm (A_{490}) of each concentration of L-citrulline for construction of standard curve

No.	Amount of L-citrulline (µmole)	Mean of A_{490}±SD
1	0.183	0.945±0.033
2	0.160	0.779±0.030
3	0.137	0.609±0.035
4	0.114	0.467±0.033
5	0.091	0.322±0.004
6	0.068	0.208±0.016
7	0.046	0.113±0.009
8	0.023	0.041±0.011
9	0.011	0.013±0.002
10	0.006	0.004±0.002

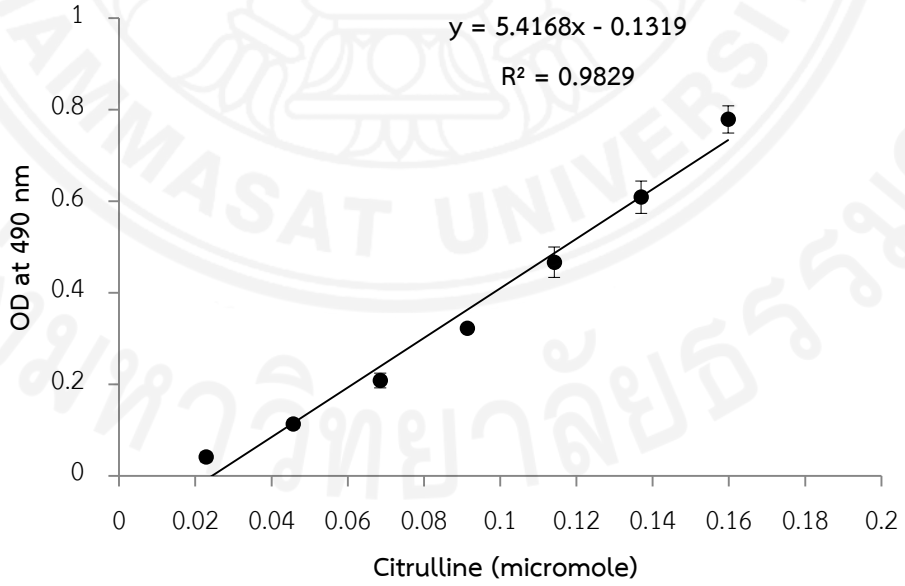


Figure 5.13 The standard curve illustrating a linear correlation between the concentrations of L-citrulline and absorbance (490 nm). The results are mean values obtained from triplicate independent experiments with error bars indicating ± SD values

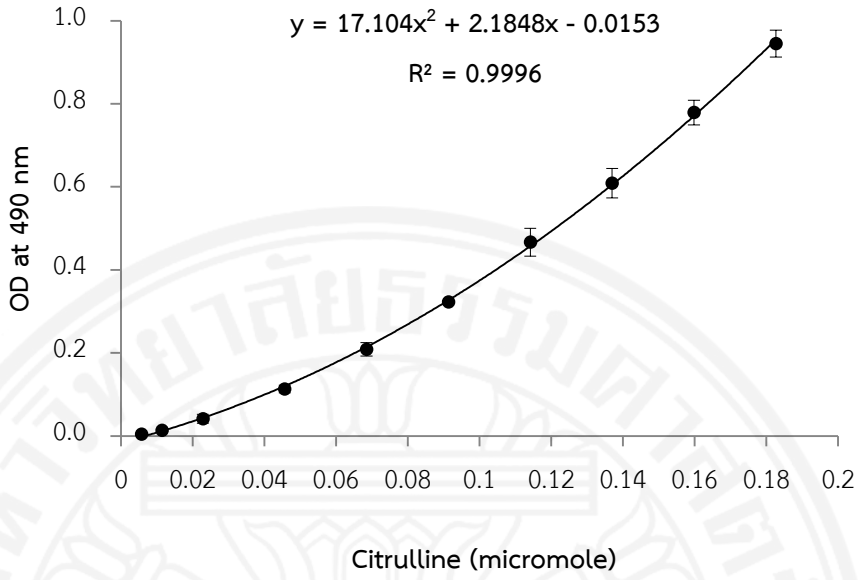


Figure 5.14 Non-linear standard curve illustrating a polynomial correlation between the concentrations of citrulline and absorbance (490 nm). The results are mean values obtained from triplicate independent experiments with error bars indicating ± SD values

5.4.3 Analysis of deduced amino acid sequence of r*SS2* ArcA

NCBI conserved domain database searching indicated that deduced amino acid sequence of r*SS2 arcA* contained four conserved motifs (FTRD, EGGDV, MHLDT and CMSxP) and a catalytic triad, Cys-His-Glu/Asp,[148] which are unique characters of superfamily of guanidine group-modifying enzymes (**Figure 5.15**). The members of this superfamily includes ArcA or ADI (EC 3.5.3.6), Arg:Gly amidinotransferase (EC 2.1.4.1), and dimethylarginine dimethylaminohydrolase (EC 3.5.3.18), respectively (**Figure 5.15**).

Multiple sequence alignment of deduced amino acid sequence of r*SS2 arcA* (**Section 4.9.9**) revealed 100% match to the ArcA of *SS2* reference strain P1/7 (NCBI Accession no. YP_003026485.1) and a *SS2* Chinese outbreak strain (NCBI Accession No. CAZ51333.1). r*SS2* ArcA exhibited amino acid sequence similarity to ArcA of *S. pyogenes* (90%), *B. cereus* (53%), *L. buchneri* NCDO001 (49%), *L. lactis*

```
S. suis HE06     -----MSNHPIHVFSEIGKLKKVMLHRPGKEIENLMPDYLERLLFDDIPFLEDAQKEHDAFAQALRDEGVEVLYLEKLAAESLVTP--EIREQFIDEYLEEAN-IRGRATKKAIRKLLMSIED 115
S. suis P1/7     -----MSNHPIHVFSEIGKLKKVMLHRPGKEIENLMPDYLERLLFDDIPFLEDAQKEHDAFAQALRDEGVEVLYLEKLAAESLVTP--EIREQFIDEYLEEAN-IRGRATKKAIRKLLMSIED 115
S. pyogenes      ----MTAQTPIHVYSEIGKLKKVLLHRPGKEIENLMPDYLERLLFDDIPFLEDAQKEHDAFAQALRDEGIEVLYLETLAAESLVTP--EIREAFIDEYLSEAN-IRGRATKKAIRELLMAIED 116
L. lactis        ------MNNGINVNSEIGKLKSVLLHRPGAEVENITPDTMKQLLFDDIPYLKIAQKEHDFFAQTLRDNGAETVYIENLATEVFEKS-SETKEEFLSHLLHEAG-YRPGRTYDGLTEYLTSMS- 114
L. buchneri      ------MTSPIHVTSEIGKLKTVLLKRPGREIENFTPDMMPRLLFDDIPYLPIAQKEHDYFADTLRDNGVEVLYLEKLAAEALDAAGDKVRNDFLEQMLTESG-YATGTIHDALKEYLGSMN- 115
M. arginini      MSVFDSKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELLFSAILESHDARKEHKQFVAELKANDINVVELIDLVAETYDLASQEAKDKLIEEFLEDSEPVLSEEHKVVVRNFLKAKKT 123
P. putida        ---MSAEKQKYGVHSEAGKLRKVMVCSPGLAHKRLTPSNCDELLFDDVIWVDQAKRDHFDFVTKMRERGVDVLEMHNLLTDIVQQP--EALKWILDRKITSDT-VGVG-LTNEVRSWLEGLE- 115
P. aeruginosa    ---MSTEKTKLGVHSEAGKLRKVMVCSPGLAHQRLTPSNCDELLFDDVIWVNQAKRDHFDFVTKMRERGIDVLEMHNLLTETIQNP--EALKWILDRKITADS-VGLG-LTSELRSWLESLE- 115
B. cereus        ------MKHPIHVTSEIGELQTVLLKRPGKEVENLTPDYLQQLLFDDIPYLPIIQKEHDYFAQTLRNRGVEVLYLEKLAAEALVDK--KLREEFVDRILKEGQ-ADVNVAHQTLKEYLLSFS- 113
E. coli          -------MEKHYVGSEIGQLRSVMLHRPNLSLKRLTPSNCQELLFDDVLSVERAGEEHDIFANTLRQQGIEVLLLTDLLTQTLDIP--EAKSWLLETQISDYR-LGPT-FATDVRTWLAEMS- 111
A. fumigatus     ------MLPPLETGRFFFPPEWHPHIATILGFPSQAATCGELYLQNCREIVDLAAAIAEFEPVRLYARPEDVPGVQQLVHRTVDDP---SRVTVIPTSINHCW------VRDTGPVYVHDASG 108

S. suis HE06     NQELVEKTMAGVQKAELPEIP-SEEKGLTDLVESSYPFAIDPMPNLYFTRDPFATIGNAVSLNHMYSETRNRETLYGKYIFTHHPEYGG-KVPLVYNREE----TTRIEGGDELVLSKDVLAV 232
S. suis P1/7     NQELVEKTMAGVQKAELPEIP-SEEKGLTDLVESSYPFAIDPMPNLYFTRDPFATIGNAVSLNHMYSETRNRETLYGKYIFTHHPEYGG-KVPLVYNREE----TTRIEGGDELVLSKDVLAV 232
S. pyogenes      NQELIEKTMAGVQKSELPEIP-ASEKGLTDLVESSYPFAIDPMPNLYFTRDPFATIGTGVSLNHMFSETRNRETLYGKYIFTHHPIYGGGKVPMVYDRNE----TTRIEGGDELVLSKDVLAV 234
L. lactis        TKDMVEKIYAGVRKNELDIKR-TALSDMAGSDAENY-FYLNPLPNAYFTRDPQASMGVGMTINKMTFPARQPESLITEYVMANHPRFKDT--PIWRDRNH----TTRIEGGDELILNKTTVAI 229
L. buchneri      NQDMVNKIMEGIRKNEVDFVP-TDLVSAA--ETENYEFYMDPMPNLYFTRDPSACIGEGLSINHMTFPARQRESLFNEIVIKYHPRFADKGVHVWRDRNH----DTRIEGGDELVLNDHVMAI 231
M. arginini      SRKLVEIMMAGITKYDLGIEA-------------DHELIVDPMPNLYFTRDPFASVGNGVTIHYMRYKVRQRETLFSRFVFSNHPKLIN---TPWYYDPS---LKLSIEGGDVFIYNNDTLVV 227
P. putida        PRHLAEFLIGGVAGQDLPVSEGAEVIKMYNKYLGHSSFILPPLPNTQFTRDTTCWIYGGVTLNPMYWPARRQETLLTTAIYKFHKEFTGADFQVWYGDPDKDHGNATLEGGDVMPVGKGIVLI 238
P. aeruginosa    PRKLAEYLIGGVAADDLPASEGADILKMYREYLGHSSFLLPPLPNTQFTRDTTCWIYGGVTLNPMYWPARRQETLLTTAIYKFHPEFANAEFEIWYGDPDKDHGSSTLEGGDVMPIGNGVVLI 238
B. cereus        NEELIQKIMGGVRKNEIETSKKTHLYELM---EDHYPFYLDPMPNLYFTRDPAASVGDGLTINKMREPARRRESLFMEYIIKYHPRFAKHNVPIWLDRDY----KFPIEGGDELILNEETIAI 229
E. coli          HRDLARHLSGGLTYSEIPASIKNMVVDTH----DINDFIMKPLPNHLFTRDTSCWIYNGVSINPMAKPARQRETNNLRAIYRWHPQFAGGEFIKYFGDENINYDHATLEGGDVLVIGRGAVLI 230
A. fumigatus     ELDPKQRLAISFEFNEWGNKN-----------------GWEGIDGDYRYANP-----------SMSPEALQENTDFARNVIESDTAPSP----VQVVKSR-----IRTEGGGLVVDGEGTLIV 194

S. suis HE06     GISQRTDAASIEKLLVNIFER-HVGFKKVLAFEFANNRKFMHLDTVFTMVDYDKFTIHPEIEG---DLRVFSVTYENDTLHIEEEH-GDLAELLAANLGLEK--VELIRCGGGDMVAAGREQW 348
S. suis P1/7     GISQRTDAASIEKLLVNIFER-HVGFKKVLAFEFANNRKFMHLDTVFTMVDYDKFTIHPEIEG---DLRVFSVTYENDTLHIEEEH-GDLAELLAANLGLEK--VELIRCGGGDMVAAGREQW 348
S. pyogenes      GISQRTDAASIEKLLVNIFKQ-NLGFKKVLAFEFANNRKFMHLDTVFTMVDYDKFTIHPEIEG---DLRVYSVTYDNEELHIVEEK-GDLAELLAANLGVEK--VDLIRCGGDNLVAAGREQW 350
L. lactis        GVSERTSSKTIQNLAKELFANPLSTFDTVLAVEIPHNHAMMHLDTVFTMINHDQFTVFPGIMDGAGNINVFILRPGKDDE-VEIEHLTDLKAALKKVLNLSE--LDLIECGAGDPIAAPREQW 349
L. buchneri      GVSQRTSADAIQDIAKSLFK--EGHFDTVIAIKIPHNHAMMHLDTVFTMINYDQFTVHPGILGEGGHIDTWTITPGKNGD-LNLDHRTDLKQVLKDALKLDD--LDLIPTGNGDPIIAGREQW 349
M. arginini      GVSERTDLQTVTLLAKNIVANKECEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPIAND---VFKFWDYDLVNGGAEPQPVENGLPLEGLLQSIINKKPVLIPIAGEGASQMEIERETH 347
P. putida        GMGERTSRHAIGQLAQNLFEK--GAAEKIIVAGLPKSRAAMHLDTVFSFCDRDLVTVFPEVVK-EIKPFIITPDSSKPYGMNIAPQDASFLEVVSEQLLGKKDKLRVVETG-GNSFAAEREQW 358
P. aeruginosa    GMGERSSRQAIGQVAQSLFAK--GAAERVIVAGLPKSRAAMHLDTVFSFCDRDLVTVFPEVVK-EIVPFSLRPDPSSPYGMNIRREEKTFLEVVAESLGLKK--LRVVETG-GNSFAAEREQW 356
B. cereus        GVSARTSAKAIERLAKNLFSR-QNKIKKVLAIEIPKCRAFMHLDTVFTMVDYDKFTIHPAIQGPKGNMNIYILEKGSDEETLKITHRTSLMEALKEVLGLSE--LVLIPCGGGDVIASAREQW 349
E. coli          GMSERTTPQGIEFLAQALFKH--RQAERVIAVELPKHRSCMHLDTVMTHIDIDTFSVYPEVVRPDVNCWTLTPDG---HGGLKRTQESTLLHAIEKALGIDQ--VRLITTG-GDAFEAEREQW 346
A. fumigatus     AESYMVCDQRNPGMSRDEIEAELRRLLGVEKVIWVPGRKGLDITDCHVDAEVRFIRPGVLVWSRHHPSVPQVWLDMSQEIRNILEDETDAKGRKFELHAIDEPGPEDLGIQEHDEFVSGYANF 317

S. suis HE06     NDGSNTLTIAPGVVVVYKRNTITNAILESKGLRLIKIGGSELVRGRGGPRCMSMPFEREDI-- 409
S. suis P1/7     NDGSNTLTIAPGVVVVYKRNTITNAILESKGLRLIKIGGSELVRGRGGPRCMSMPFEREDI-- 409
S. pyogenes      NDGSNTLTIAPGVVVVYNRNTITNAILESKGLKLIKIHGSELVRGRGGPRCMSMPFEREDI-- 411
L. lactis        NDGSNTLAIAPGEIVTYDRNYVTVELLKEHGIKVHEILSSELGRGRGGARCMSQPLWREDL-- 410
L. buchneri      NDGSNTLAIAPGVVVTYNRNYVSNDLLRKHGLKVLEVISSELSRGRGGPRCMSCPIVREDI-- 410
M. arginini      FDGTNYLAIRPGVVIGYSRNEKTNAALEAAGIKVLPFHGNQLSLGMGNARCMSMPLSRKDVKW 410
P. putida        DDGNNVVALEPGVVIGYDRNTYTNTLLRKAGIEVITISAGELGRGRGGGHCMTCPIVRDPIDY 420
P. aeruginosa    DDGNNVVCLEPGVVVGYDRNTYTNTLLRKAGVEVITISASELGRGRGGGHCMTCPIVRDPIDY 418
B. cereus        NDGSNTLAIAPGVVVTYDRNYVSNTLLREHGIEVIEVLSSELSRGRGGPRCMSMPIVRKDI-- 410
E. coli          NDANNVLTLRPGVVVGYERNIWTNEKYDKAGITVLPIPGDELGRGRGGARCMSCPLHRDGI-- 406
A.fumigatus      YFCNGGVIVPGFGVEEYDRKARETLQALMPERKVRQVQLNAIPLSGGVIHCVTQQIPMPAA-- 378
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(Please see overleaf for figure legend)

Figure 5.15 Multiple alignments of deduced amino acid sequences of ArcA. The consensus sequence from HE06 *S. suis* (This study) was analyzed by comparing with other organisms. Four highly conserved sequence motifs in guanidine-group-modifying enzymes are colored in gray boxes. The residues contributing in catalysis are indicated by underline (_). ArcA amino acid residues of other organisms: *S. suis* P1/7 (GenBank accession no. CAR45282.1), *S. pyogenes* (GenBank accession no. AFC68424.1), *L. lactis* (GenBank accession no. DQ364637), *L. buchneri* (GenBank accession no. WP_014939465.1), *M. arginini* (GenBank accession no. CAA38210.1), *P. putida* (GenBank accession no. AAA16964.1), *P. aeruginosa* (GenBank accession no. WP_023108863.1), *B. cereus* (GenBank accession no. AAP07446.1), *E. coli* (GenBank accession no. AIL18158.1), *A. fumigatus* (GenBank accession no. XP_747453.1)

ssp. *lactis* ATCC 7962 (46%), *E. coli* (36%), *M. arginini* (36%), *P. putida* (32%), *P. aeruginosa* (32%), and *A. fumigatus* (7%) (**Figure 5.15** and **Table 5.6**). Among these orthologues, the three-dimensional structure of *Mycoplasma* ArcA and *Pseudomonas* ArcA have been clearly elucidated and the catalytic reaction mechanism of ArcA has been proposed.[33,148] The multiple amino acid sequence alignments demonstrated a highly conserved catalytic triad of *SS2* ArcA, Cys399-His273-Glu218 that was equivalent to Cys398-His269-Glu213 of *M. arginini* ArcA, and a major residue involved in the reaction mechanism of *SS2* ArcA was Cys399 (**Figure 5.15**).

5.4.4 Expression and purification of r*SS2* ArcA

E2096 *E. coli* clone carrying pET23a+*::arcA* was successfully induced to express the soluble r*SS2* ArcA (**Lane 3**, **Figure 5.12**). For large scale purification of the rArcA, log-phase culture of E2096 *E. coli* clone carrying pET23a+*::arcA* (350 ml) was induced by shifting temperature up to 42 ºC and incubated further as described in **Sections 5.4.1** and **4.9.10**. The cells were harvested by centrifugation and soluble rArcA was purified by using Ni-NTA agarose affinity chromatography (Invitrogen) as described in **Section 4.9.11.** According to the inactivation of r*SS2* ArcA by imidazole (data not shown), the EDTA elution buffer was applied in this study. Initially, approximately 184.1 mg of crude extract were obtained from 350-ml culture of *E. coli* clone and yielded total ArcA activity of 11.7 U but due to it unpurified nature a low specific activity at 0.06 U mg^{-1} was obtained (**Table 5.7**). For r*SS2* ArcA eluted from the nickel chromatographic column using EDTA elution buffer, after protein binding and washing until no protein presence, the stepwise of EDTA elutions were further conducted. Purification profile of r*SS2* ArcA was shown in **Figure 5.16**. Fractions no. 1–9 and 10–22 were eluted by 0.1 mM and 0.25 mM EDTA, respectively, containing numerous contaminant proteins without ArcA activity (**Figure 5.16A**). Fractions no. 23–29 eluted with 0.5 mM EDTA also exhibited many of contaminant proteins (**Figure 5.16B**). A minimum ArcA specific activity was observed in fraction no. 28 (0.27 U/mg) and 29 (0.80 U/mg) (**Figure 5.16A**). Fractions no. 30–40 were eluted with 1.5 mM EDTA yielding high ArcA specific activity (2.41, 4.14, 4.61, 7.64, 10.54, 14.46, 19.39, 21.59, 20.71, 14.11, 9.39 U/mg, respectively) (**Figure 5.16A**). As shown in SDS-PAGE and Western-blot analysis, fractions no. 34–40 exhibited the

Table 5.6 Amino acid identity matrix of *SS2* ArcA and their orthologues

Organism(s)	No. of amino acids	% amino acid identities of orthologues ArcA										
		HE06 *S. suis*	P1/7 *S. suis*	*S. pyogenes*	*L. lactis*	*L. buchneri*	*M. arginini*	*P. putida*	*P. aeruginosa*	*B. cereus*	*E. coli*	*A. fumigatus*
HE06 *S. suis*	409	100.00	100.00	89.98	45.72	48.90	35.70	31.78	32.03	53.06	35.96	7.14
P1/7 *S. suis*	409	100.00	100.00	89.98								
S. pyogenes	411	89.98	89.98	100.00								
L. lactis	410	45.72	45.72	45.61	100.00							
L. buchneri	410	48.90	48.90	49.02	60.24	100.00						
M. arginine	410	35.70	35.70	38.05	34.88	33.90	100.00					
P. putida	420	31.78	31.78	31.63	30.73	31.22	27.07	100.00				
P. aeruginosa	418	32.03	32.03	32.85	31.71	32.20	27.32	84.21	100.00			
B. cereus	410	53.06	53.06	51.71	56.59	60.49	35.12	84.39	35.12	100.00		
E. coli	406	35.96	35.96	36.95	36.21	38.18	30.54	46.80	48.03	36.45	100.00	
A. fumigatus	378	7.14	7.14	2.91	7.67	6.35	10.85	8.73	9.52	5.56	5.03	100.00

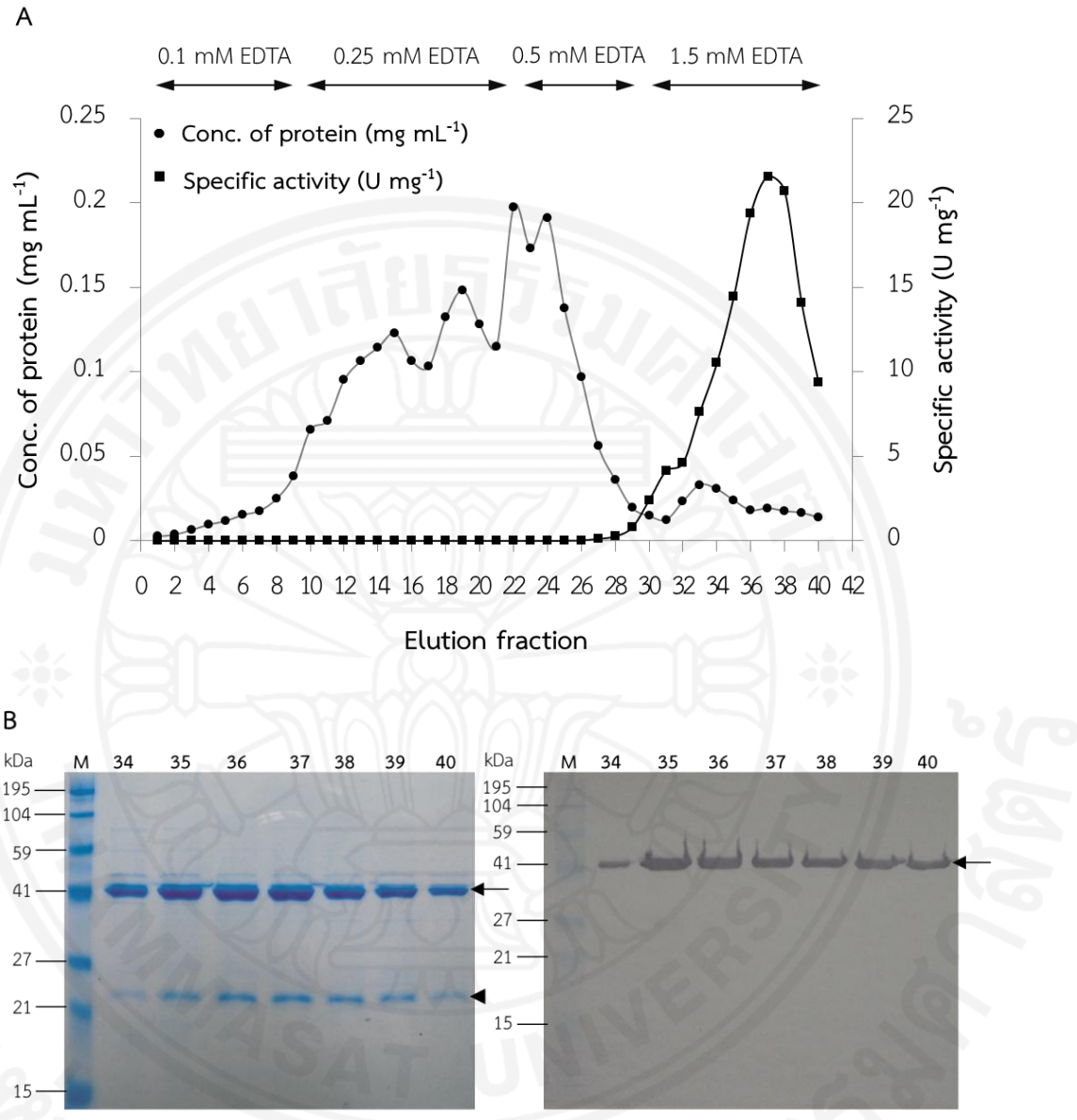

Figure 5.16 The purification profile of r*SS2* ArcA. The r*SS2* ArcA was purified using Ni-NTA agarose affinity chromatography and EDTA-gradient elution. (A) The chromatogram represents the purification profile of r*SS2* ArcA. The gray and black lines represent the protein concentration and the specific activity of each EDTA-eluted fraction, respectively. (B) The SDS-PAGE and Western blot analysis of EDTA-eluted fractions no. 34–40. The numbers labeled in the x-axis of chromatogram are numbers of elution fractions which accompanied with the numbers labeled on top of the SDS-PAGE and Western blot figures. Arrows indicate 47 kDa of purified r*SS2* ArcA. Arrow head indicates C-terminal truncated r*SS2* ArcA (*ca*. 25 kDa)

Table 5.7 Expression and purification of r*SS2* ArcA in E2096 *E. coli*

Purification step	Volume (ml)	Conc. of protein (mg/ml)	Activity (U)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold of purification
Crude extract	5	36.82	2.34	184.1	11.7	0.06	100	–
Last washing fraction	5	ND	0	ND	0	0	0	0
Elution fraction (Fractions No. 35–39)	12.5	0.02	0.32	0.25	4.00	16.00	34.19	266.67

ND denotes “not determined”

r*SS2* ArcA protein was prepared from 350 ml of induced *E. coli* culture.

Enzymatic activity defined as Unit (U) = µmoles/min/ml

% yield = (Total activity at any step/Total activity at the first step) × 100

Fold of purification = Specific activity at any step/Specific activity at the first step

purity of r*SS2* ArcA. The highest purity with ArcA specific activity was observed in fractions no. 35–39 (14.46, 19.39, 21.59, 20.71, 14.11 U/mg, respectively), which were then pooled and used further for testing physical properties and enzyme kinetics. In addition, the purified r*SS2* ArcA was verified by mass spectrometry. The 47 kDa protein band of purified r*SS2* ArcA was excised from SDS-PAGE gel and trypsin-digested (**Section 4.9.12**). The mixture was then subjected to LC-MS/MS analysis. Matched peptides from LC-MS/MS analysis indicated that 129 peptides containing 8–17 amino acid residues, matched to the peptide sequences of *SS2* ArcA [*SS2* 89/1591] Accession no. 223933313 (**Table 5.8**, **Appendix E**). The result confirmed that the purified recombinant protein obtained in this study was ArcA of *SS2*. In addition, 25 kDa of protein band as shown in SDS-PAGE was also tested by immunoblot using mouse anti-ArcA sera and LC-MS/MS analysis. This band was ArcA of serotype 2 *S. suis* but it degraded from C-terminal part, thus it could not react by using murine anti-His_6-tag antibodies.

The efficiency of purification method for r*SS2* ArcA using E2096 *E. coli* system was summarized in a purification table (**Table 5.7**). According to the inactivation of r*SS2* ArcA by imidazole, the EDTA elution buffer was applied in this study. At 0.5 mM EDTA elution step, the r*SS2* ArcA was co-eluted with contaminant proteins and fractions no. 30–34 and 40 containing some contaminant proteins were excluded from the study. As a result, the yield of protein purification was somewhat low (34.19%). However, this single-step affinity-based purification procedure provided a high purity (266.67-fold purification) yielding 0.25 mg of the purest r*SS2* ArcA protein (pooled fractions no. 35–39) with total activity and specific activity of ArcA as 4.00 U and 16.00 U/mg, respectively.

5.4.5 Stability test of purified r*SS2* ArcA

The purified r*SS2* ArcA was preserved in 20% glycerol (v/v) at −80 ºC (**Section 4.9.11**) and the stability of enzymatic activity was then determined by conducting the ArcA activity assay monthly for six months (**Section 4.11.2**). The results of stability are shown in **Figure 5.17**. The results indicated that r*SS2* ArcA could be stored and stably maintained the original enzymatic activity for at least six months at −80 ºC.

Table 5.8 Orthologous proteins of the database containing peptide sequence(s) matched with peptides generated from the Ni-NTA column purified r*SS2* ArcA in the gel plug excised from the SDS-PAGE gel

Band No.	Matched protein	Accession No.	Score	Coverage ΔCn	Matched peptides shown in bold
1	Arginine deiminase [*S. suis* strain 89/1591]	gi|223933313	250.22	40.83	MSNHPIHVFSEIGKLKKVMLHRPG**KEIENLMPDYLERL**LFDDIPF LEDAQKEHDAFAQAL**RDEGVEVLYLEKLAAESLVTPEIRE**QFIDE YLEEANIRGRATKKAIRKLLMSIEDNQELVEKTMAGVQKAELPEI PSEEKGLTDLVESSYPFAIDPMPNLYFTRDPFATIGNAVSLNHMY SET**RNRETLYGKYIFTHHPEYGGKVPLVYNREETTRIEGGDELVL SKDVLAVGISQRTDAASIEKL**LVNIFERHVGF**KKVLAFEFANNRK F**MHLDTVFTMVDYD**KFTIHPEIEGDLRV**FSVTYENDTLHIEEEHG DLAELLAANLGLEKVELI**RCGGGDMVAAGRE**QWNDGSNTLTIAPG VVVVY**KRNTITNAILESKG**LRLIKIGGSELVRGRGGP**RCMSMPFE RE**DI
2	Arginine deiminase [*S. suis* strain 89/1591]	gi|223933313	104.28	27.87	**MSNHPIHVFSEIGKL**KKVMLHRPGKEIENLMPDYLERLLFDDIPF LEDAQKEHDAFAQAL**RDEGVEVLYLEKLAAESLVTPEIREQFIDE YLEEANIRG**RATKKAIRKLLMSIEDNQELVEKTMAGVQKAELPEI PSEEKGLTDLVESSYPFAIDPMPNLYFT**RDPFATIGNAVSLNHMY SETRN**RETLYGKYIFTHHPEYGG**KVPLVYNREETTRI**EGGDELVL SKDVLAVGISQRTDAASIEKLLVNIFERHVGFKKVLAFEFANNRK FMHLDTVFTMVDYDKFTIHPEIEGDLRVFSVTYENDTLHIEEEHG DLAELLAANLGLEKVELI**RCGGGDMVAAGRE**QWNDGSNTLTIAPG VVVVY**KRNTITNAILESKG**LRLI**KIGGSELVRG**RGGPRCMSMPFE REDI

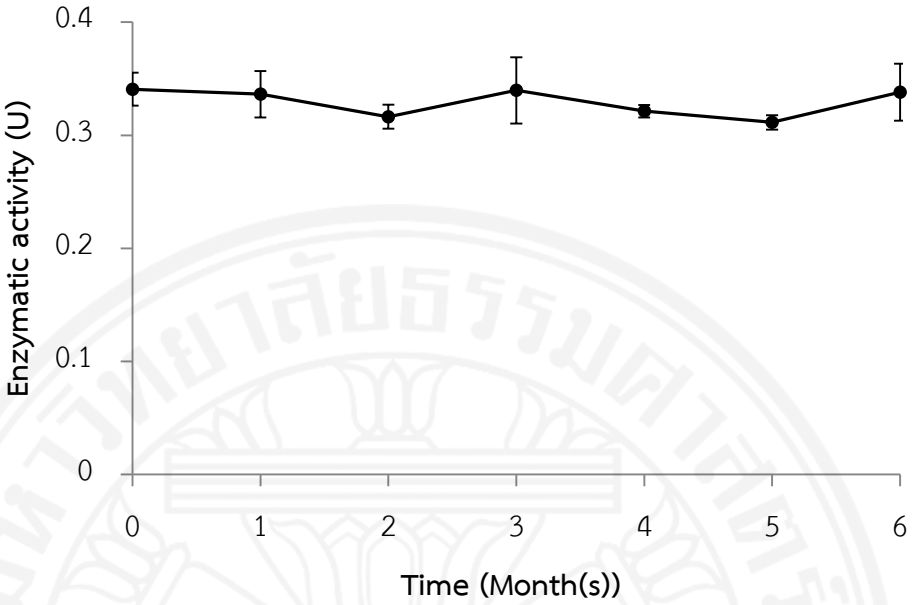

Figure 5.17 Enzymatic activity of r*SS2* ArcA protein in the six-month stability test at −80 ºC. The results are mean values obtained from triplicate assays with error bars indicating ± SD values

5.4.6 Production of mouse anti-r*SS2* ArcA polyclonal antibody

Three outbred ICR mice were immunized intraperitoneally with of three 10-µg doses of purified r*SS2* ArcA in Alum adjuvant (**Section 4.9.13**). Blood was collected by cardiac puncture one week after the last immunization, and immune sera were kept in small aliquots at −20 ºC. The indirect ELISA titer of immune sera was 32,000 (**Section 4.9.13**). Immune sera with the 1:8,000 dilutions were used further in Western immunoblot analysis.

5.4.7 Determination of physical properties of r*SS2* ArcA

5.4.7.1 Temperature dependency of r*SS2* ArcA activity

The optimal temperature for r*SS2* ArcA activity was determined by conducting the ArcA activity assay (**Section 4.11.2**) at different temperatures including, 25, 30, 37, 42, 50, 60 and 70 ºC. The results are shown in **Table 5.9** and **Figure 5.18** indicating that r*SS2* ArcA had low enzymatic activity at 25 and 30 ºC and significant enzymatic activity was found at 37 ºC and the optimum

Table 5.9 Temperature dependency of r*SS2* ArcA activity

Temperature (°C)	Mean of enzymatic activity±SD (U)
25	0.133±0.015
30	0.202±0.012
37	0.332±0.004
42	0.392±0.013
50	0.152±0.006
60	0.045±0.002
70	0.025±0.002

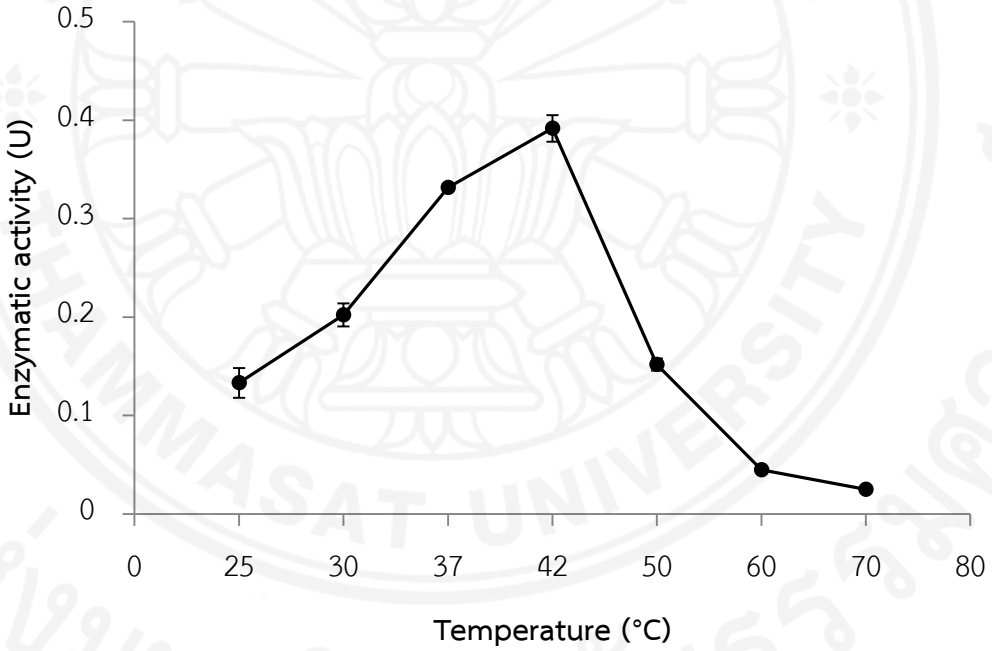

Figure 5.18 Temperature dependency of r*SS2* ArcA activity. The results are mean values obtained from triplicate independent experiments with error bars indicating ± SD values

temperature of r*SS2* ArcA activity was 42 ºC. However, at temperatures higher than 42 ºC, the r*SS2* ArcA activity rapidly decreased.

5.4.7.2 pH dependency of r*SS2* ArcA activity

An optimal pH for r*SS2* ArcA activity was determined by conducting the ArcA activity assay (**Section 4.11.2**) in different pH of assay buffer, ranging from pH 4.2 to 8.0. As shown in **Table 5.10** and **Figure 5.19**, the enzymatic activity of r*SS2* ArcA was decreased when the pH was higher than 7.2 and drastically diminished at pH 8.0, indicating alkaline pH sensitivity of the r*SS2* ArcA activity. As shown in **Table 5.14**, the ArcA has an optimal pH in neutral or slightly acidic condition. These results indicated that r*SS2* ArcA could functionally active in a wide range of pH between 4.2 and 7.5 suggesting the ArcA of *S. suis* prefers the acidic environment for its function.

5.4.7.3 Temperature stability of r*SS2* ArcA

Thermostability of r*SS2* ArcA was determined by pre-incubating purified r*SS2* ArcA at 4, 25, 30, 37 and 42 ºC for 3 h. The pre-incubated r*SS2* ArcA at a particular temperature was taken at every 30 min intervals for the ArcA activity assay (**Section 4.11.2**). The results shown in **Table 5.11** and **Figure 5.20** indicated the stability of r*SS2* ArcA activities at 4 and 25 ºC for 3 h. The r*SS2* ArcA activity remained approximately 80% of the original activity for 2 h at 30 ºC. At 37 ºC pre-incubation, the r*SS2* ArcA activity reduced to 50% within an hour and completely diminished after 2 h of pre-incubation. At 42 ºC pre-incubation, the activity of r*SS2* ArcA remained 50% within 30 min and abolished after 2 h of pre-incubation. All of these results indicated that the r*SS2* ArcA was temperature sensitive when pre-incubation without L-arginine substrate at 37 ºC and 42 ºC, although, their optimum temperature for the activity r*SS2* was noted at these temperatures (**Section 5.2.7.1**, **Table 5.9** and **Figure 5.18**).

5.4.7.4 pH stability of r*SS2* ArcA

The pH stability of r*SS2* ArcA was determined by pre-incubating purified r*SS2* ArcA enzyme in 100 mM potassium phosphate buffer (assay buffer) at different pH (pH 4.2, 5.0, 6.0, 7.2 and 8.0). The pre-incubation was conducted at 25, 30 and 37 ºC for 3 h. The ArcA activity was determined under the ArcA assay condition at 37 ºC and pH 7.2 (**Section 4.11.2**). As shown in **Table 5.12**

Table 5.10 pH dependency of r*SS2* ArcA activity

pH	Mean of enzymatic activity±SD (U)
4.2	0.116±0.002
4.5	0.131±0.006
5.0	0.140±0.005
5.5	0.176±0.003
6.0	0.220±0.004
6.2	0.243±0.008
6.5	0.315±0.005
7.2	0.329±0.004
7.5	0.203±0.011
8.0	0.028±0.004

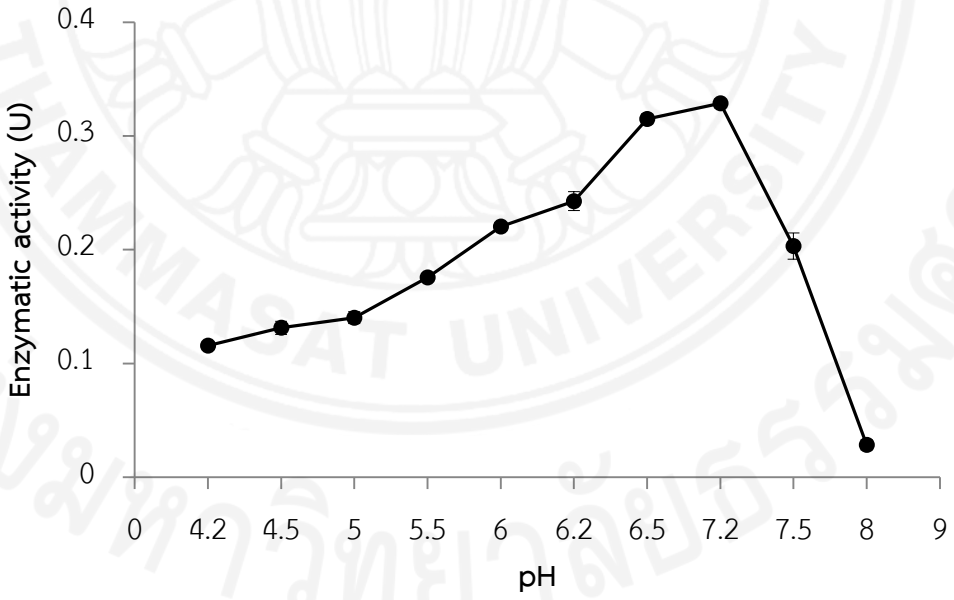

Figure 5.19 pH dependency of ArcA activity. The results are mean values obtained from triplicate independent experiments with error bars indicating ± SD values

Table 5.11 Temperature stability of r*SS2* ArcA

Temperature (°C)	Mean of enzymatic activity±SD (U) at indicated pre-incubation time						
	0 min	30 min	60 min	90 min	120 min	150 min	180 min
4	0.316± 0.001	0.313± 0.008	0.312± 0.001	0.309± 0.009	0.307± 0.007	0.297± 0.009	0.297± 0.007
25	0.316± 0.001	0.310± 0.006	0.304± 0.003	0.304± 0.004	0.293± 0.003	0.294± 0.003	0.291± 0.004
30	0.316± 0.001	0.296± 0.005	0.277± 0.006	0.271± 0.001	0.268± 0.009	0.236± 0.006	0.226± 0.008
37	0.316± 0.001	0.198± 0.004	0.132± 0.007	0.049± 0.004	0.027± 0.002	0.026± 0.003	0.024± 0.001
42	0.316± 0.001	0.120± 0.009	0.053± 0.005	0.034± 0.002	0.031± 0.002	0.027± 0.001	0.024± 0.002

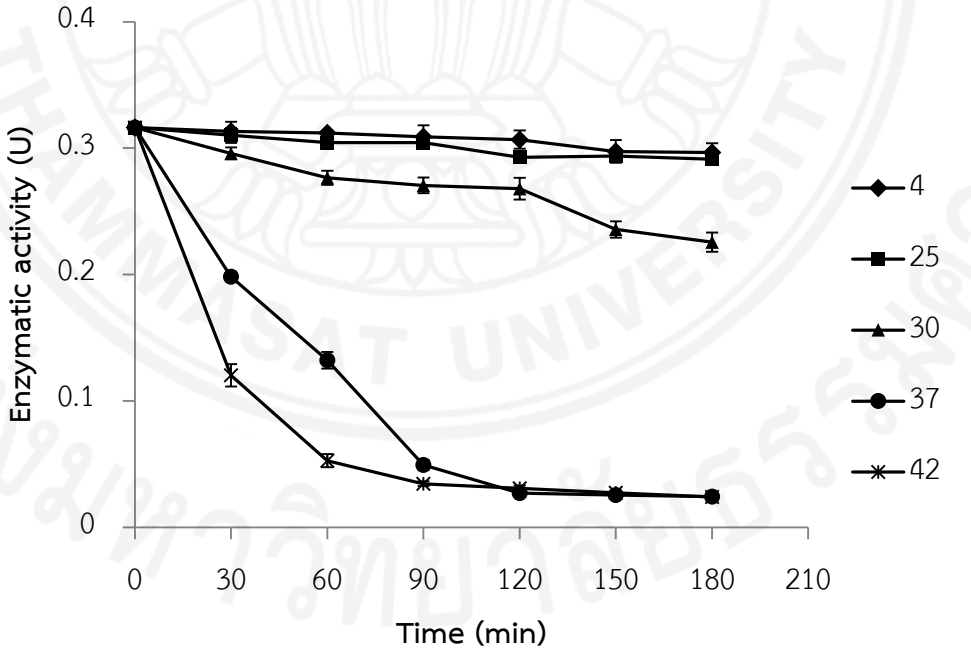

Figure 5.20 Temperature stability of r*SS2* ArcA. The enzyme solution was pre-incubated at 4 °C (diamond), 25 °C (square), 30 °C (triangle), 37 °C (circle) and 42 °C (asterisk) for 3 h. Every 30 min of incubation, the enzyme was taken for enzymatic assay. The results are mean values obtained from triplicate independent experiments with error bars indicating ± SD values

and **Figure 5.21**, the result demonstrated that the r*SS2* ArcA was stable at pH 4.2 to 6.0, especially at 25 and 30 ºC pre-incubations. The result of this experiment revealed the contradiction to that of pH dependency (**Section 5.3.7.2** and **Table 5.10**). As shown in temperature dependency and temperature stability based on the effect of L-arginine substrate binding with ArcA enzyme, this enzyme had optimum pH at 7.2 but it could not be stable at pH 7.2 without L-arginine. Interestingly, the enzymatic activity of *SS2* ArcA could be reduced after pre-incubation at 37 ºC, but it was more stable at 37 ºC under acidic condition than alkaline condition though pre-incubating without L-arginine substrate.

5.4.8 Kinetic characterization of r*SS2* ArcA

To determine the enzymatic properties of r*SS2* ArcA, Michaelis–Menten constants (K_m) and maximum velocity (V_{max}) were determined (**Section 4.11.3**) using L-arginine (0–8 mM) as a substrate at 42 ºC (temperature giving the highest r*SS2* ArcA acitivity) (**Section 5.4.7.1**) and 37 ºC (temperature for standard ArcA assay[149] (**Section 4.11.2**)**.** Enzymatic activity of the individual concentrations of L-arginine substrate for determining K_m and V_{max} of r*SS2* ArcA are shown in **Table 5.13**. The initial velocity (v_i) of r*SS2* ArcA at a specified substrate concentration (ranging from 0.008 to 8.0 mM) was measured and plotted against the substrate concentration to generate Michaelis Menten plots, as shown in **Figure 5.22**. By using non-linear regression analysis, the K_m and V_{max} values of r*SS2* ArcA at 42 ºC were calculated as 0.058 mM and 0.38 U, respectively. In addition, the K_m and V_{max} values of r*SS2* ArcA at 37 ºC were calculated as 0.020 mM and 0.27 U, respectively. According to the kinetic values of ArcA orthologues from different species as seen in **Table 5.14**, indicating that a substrate binding affinity of ArcA was highly variable among different species. Although the amino acid sequence alignment revealed a high sequence similarity at conserved regions and catalytic site of r*SS2* ArcA and that of *S. pyogenes* ArcA (**Figure 5.17**), their K_m values were significantly different, suggesting that the structural integrity of non-conserved regions might contribute to the substrate binding affinity.

Table 5.12 pH stability of r*SS2* ArcA

Pre-incubation pH	Mean of enzymatic activity±SD (U) at the indicated temperature following pre-incubation		
	25 °C	30 °C	37 °C
4.2	0.276±0.009	0.274±0.001	0.231±0.003
5.0	0.306±0.008	0.296±0.002	0.281±0.001
6.0	0.265±0.005	0.241±0.013	0.162±0.006
7.2	0.126±0.004	0.127±0.012	0.056±0.006
8.0	0.097±0.017	0.082±0.002	0.042±0.001

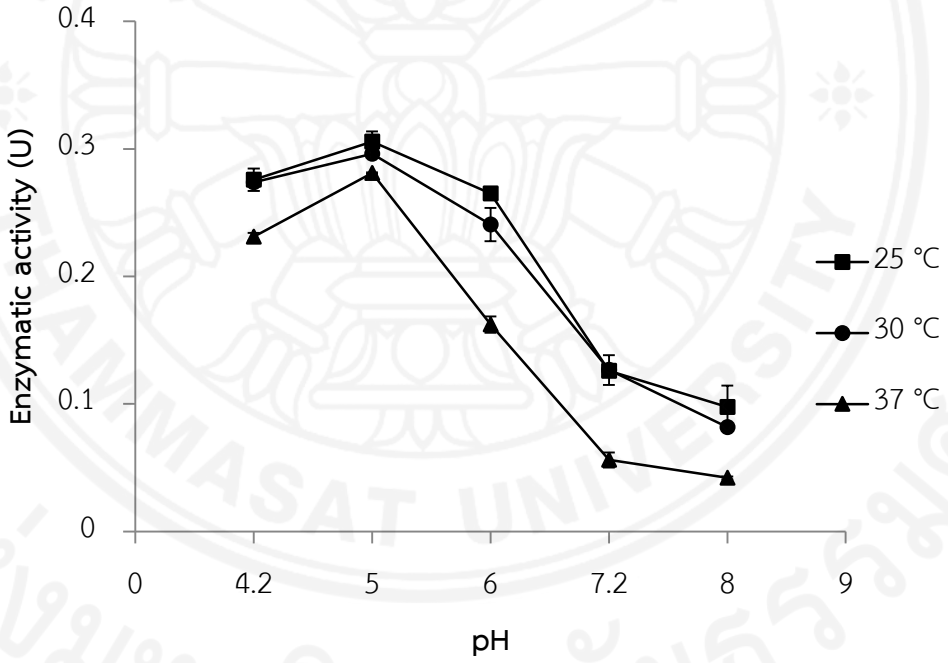

Figure 5.21 The pH stability of r*SS2* ArcA was determined by pre-incubating r*SS2* ArcA enzyme in potassium phosphate buffer at each pH (Assay buffer pH 4.2, 5.0, 6.0, 7.2 and 8.0). These reactions were conducted at 25 (square), 30 (circle) and 37 °C (triangle) for 3 h and next followed by ArcA assay. The results are mean values obtained from triplicate independent experiments with error bars indicating ± SD values

Table 5.13 Enzymatic activity of each concentration of L-arginine substrate for determining K_m and V_{max} values of r*SS2* ArcA

L-arginine	Mean of enzymatic activity±SD (U)	
(mM)	42 °C	37 °C
8.0	0.375±0.001	0.270±0.002
4.0	0.372±0.002	0.270±0.001
2.0	0.375±0.001	0.270±0.000
1.0	0.372±0.001	0.264±0.001
0.5	0.345±0.003	0.261±0.000
0.25	0.309±0.003	0.243±0.002
0.125	0.267±0.002	0.213±0.006
0.063	0.189±0.005	0.195±0.009
0.031	0.114±0.002	0.159±0.002
0.018	0.093±0.002	0.114±0.002
0.008	0.078±0.001	0.087±0.001

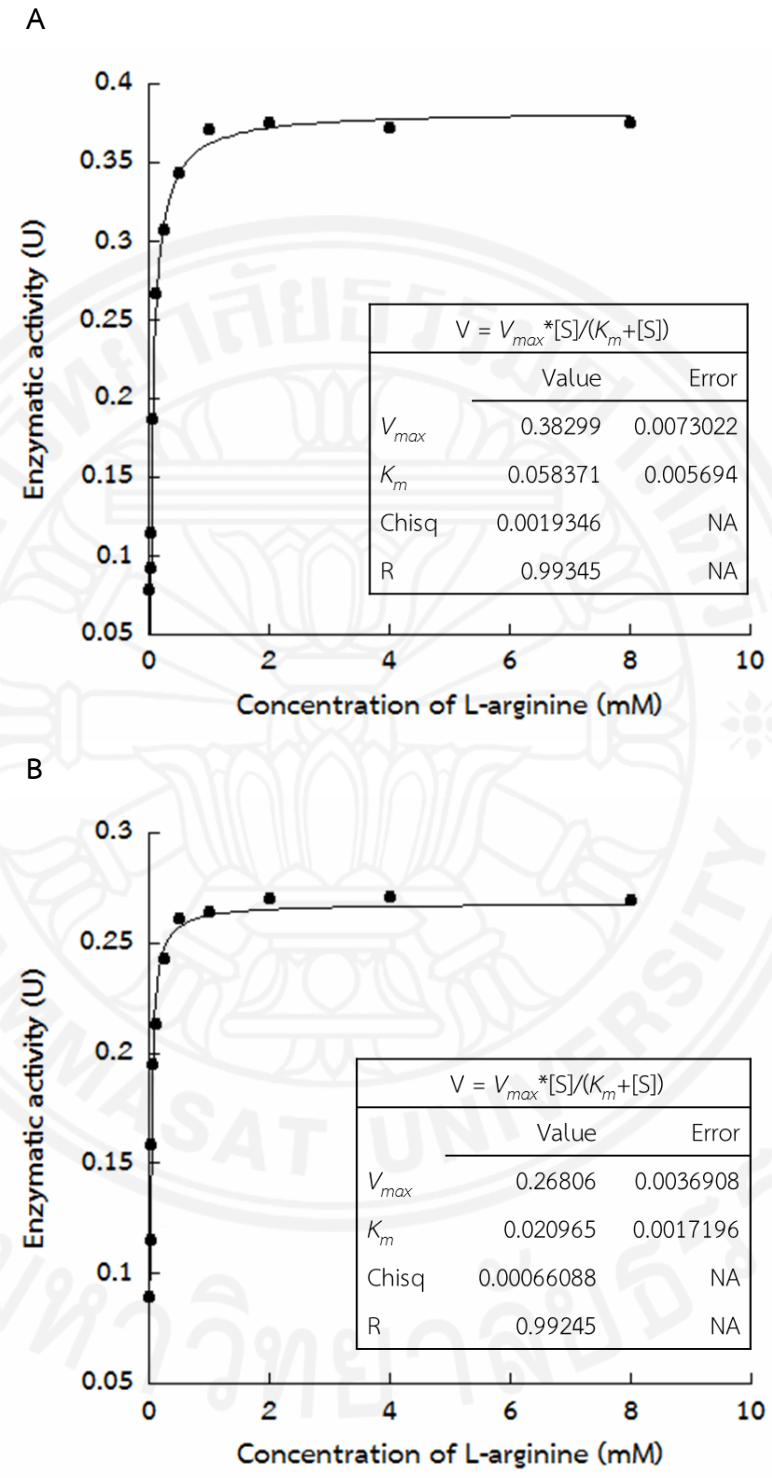

Figure 5.22 Michaelis-Menten Plot. L-arginine substrate concentrations *versus* enzymatic activity of r*SS2* ArcA. K_m and V_{max} as shown in this plot (A) Assay was conducted at 42 ºC and (B) Assay was conducted at 37 ºC. The results are mean values obtained from triplicate independent experiments

5.4.9 ArcA inhibition assay

5.4.9.1 L-canavanine inhibition of r*SS2* ArcA

L-canavanine has been used as a potent inhibitor for ArcA in many organisms such as *P. aeruginosa* , *E. coli*, *Burkholderia mallei*, *B. cereus* and *Giardia intestinalis*.[154] The ArcA inhibition by L-canavanine in different species was summarized in **Table 5.14**. Due to the restriction for water-solubility of L-canavanine, the maximum concentration used was 20 mM. As a consequence of instability of r*SS2* ArcA in its diluted form, the inhibition assay was conducted using 0.3 U of r*SS2* ArcA (referring to V_{max} value in **Section 5.4.8**) (**Section 4.11.4**). The 0.3 U of r*SS2* ArcA was pre-incubated with 20 mM L-canavanine at 25 ºC for 15 min prior to the addition of 10 mM L-arginine substrate and following by incubation at 37 ºC for the indicated time points (*i. e.*, 15, 30, 60 and 120 min). The result showed that the r*SS2* ArcA was highest inhibited by 20 mM of L-canavanine after 120 min incubation about 56.61% (**Table 5.15** and **Figure 5.23**). In addition, the inhibition was time-dependent as shown in **Table 5.15** and temperature-dependent (Pre-incubation at 0 ºC for 15 min, the r*SS2* ArcA was inhibited by 20 mM of L-canavanine about ~36% but ArcA activity lost more than 10% after pre-incubated with L-canavanine at 37 ºC for 15 min. These results were in agreement with the previous finding in this thesis that free form of r*SS2* ArcA when lacking of L-arginine substrate was thermosensitive. In this study, the increasing L-canavanine concentration to yield 100% enzyme inhibition was unachievable due to the limitation on inhibitor solubility, therefore, inhibition constant (K_i) for r*SS2* ArcA could not be carried out. However, the results of only around half of enzyme activity had been inhibited when using two times higher concentration of inhibitor over the L-arginine substrate optimum concentration suggesting that the r*SS2* ArcA had a weak binding affinity to L-canavanine inhibitor.

5.4.9.2 L-NIO inhibition of r*SS2* ArcA

L-NIO was the most potent inhibitor for NO synthase in phagocytic cells such as neutrophils and macrophages.[156] As a consequence of NO synthase has L-arginine as substrate similar to ArcA, thus L-NIO was also used for further testing in inhibition assay of r*SS2* ArcA. This inhibitor also possessed the restriction of water-solubility, so the maximum concentration of L-NIO used for the

Table 5.14 Comparison of enzymatic properties of r*SS2* ArcA and ArcA from other bacterial species

Organism	MW (kDa)	Optimum pH	Optimum temp. (°C)	K_m for L-arginine (mM)	K_i (mM)	References
***S. suis* HE06**	47	7.2	42	0.058±0.01	ND	This study
***S. pyogenes* M49 591**	47.2	6.5	37	1.33±0.12	No data	[150]
***L. lactis* ssp. *lactis* ATCC 7962**	46	7.2	60	8.67±0.045	No data	[151]
***L. buchneri* NCDO110**	46.1	6.0	50	0.83	No data	[151,152]
M. arginini	45	6.4	41	0.37±0.05	No data	[112]
P. putida	54	6.0	50	0.20	No data	[153]
P. aeruginosa	46	5.6	25	0.14±0.01	1.7±0.5	[154]
B. cereus	46.9	7.0	25	0.09±0.01	5.0±1.0	[154]
E. coli	45.9	6.0	25	0.32±0.20	5.8±0.7	[154]
A. fumigatus	50	No data	No data	8.76±1.1	No data	[155]
B. mallei	45.92	5.6	25	0.09±0.01	2.2±0.2	[154]
G. intestinalis	66	7.5	25	0.16±0.01	2.2±0.2	[154]

ND denotes “not determined”

Table 5.15 Inhibition of ArcA activity using 20 mM L-canavanine

Time (min)	Enzymatic activity (U)		Inhibition (%)
	No inhibitor	L-canavanine	
15	0.076±0.005	0.074±0.007	2.63
30	0.171±0.003	0.153±0.005	10.53
60	0.208±0.004	0.125±0.006	39.90
120	0.295±0.006	0.128±0.005	56.61

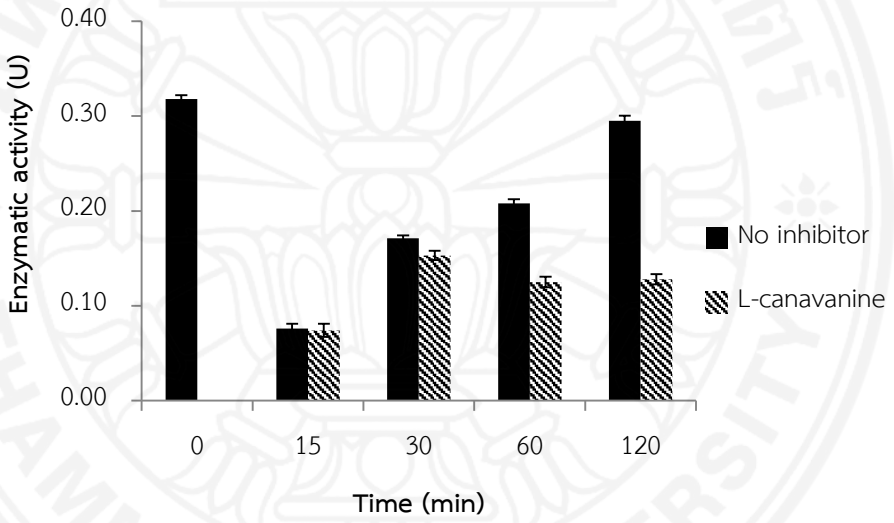

Figure 5.23 The inhibition of ArcA activity using L-canavanine. The results are mean values obtained from triplicate independent experiments with error bars indicating ± SD values

inhibition assay was 0.5 mM. Therefore, the inhibition assay of r*SS2* ArcA with L-NIO was conducted using 0.3 U of r*SS2* ArcA and 0.5 mM of L-NIO as described in **Section 4.11.4.** The result showed that the r*SS2* ArcA was inhibited by 0.5 mM of L-NIO less than 50% (44.44%) (**Table 5.16**). For this reason, K_i of r*SS2* ArcA using L-NIO also could not be determined which is similar to the use L-canavanine inhibitor. Therefore, similar to L-canavanine, this result also suggested that the r*SS2* ArcA had a weak binding affinity to L-NIO inhibitor.

Table 5.16 Inhibition assay of r*SS2* ArcA

Reaction mixture	Enzymatic activity (U)	Inhibition (%)
r*SS2* ArcA original activity		
0.3 U r*SS2* ArcA + 10 mM L-arginine	0.317±0.020	–
Negative inhibition control		
0.3 U r*SS2* ArcA + UDW* + 10 mM L-arginine	0.306±0.017	–
Positive inhibition control		
0.3 U r*SS2* ArcA + 20mM L-canavanine + 10 mM L-arginine	0.128±0.005	59.62
L-NIO inhibitory test		
0.3 U r*SS2* ArcA + 0.5 mM L-NIO + 10 mM L-arginine	0.170±0.018	44.44

*UDW represents a reagent blank because each inhibitor was dissolved in UDW

5.5 Characterization of crude native *SS2* ArcA

5.5.1. Expression of crude native *SS2* ArcA

To induce crude native *SS2* ArcA expression *in vitro, SS2* strain HE06 were grown in low glucose, Dulbecco's Modified Eagle's Medium (DMEM), lacking L-arginine (Sigma), supplemented with 10 mM L-arginine at 37 ºC, in the presence of 5% CO_2 for 2, 4, 6 and 18 h. At the indicated time points, the cultures were collected and adjusted to be equivalent to *ca*. 5×10^8 CFU/ml (OD_{600} equal to 0.5) for preparing whole cell lysate samples. Lysates were then examined by Western blot analysis using mouse anti-r*SS2* ArcA antiserum (**Section 4.10.2**). The result showed that in lysate of HE06 *SS2* grown in DMEM lacking L-arginine, antiserum weakly labelled a single band of 47 kDa protein species corresponding to expected

size of *SS2* ArcA. However, in the presence of L-arginine in culture medium, the intensity of protein band was high, thus, the expression of crude native *SS2* ArcA was successfully induced using L-arginine, as an inducer (**Figure 5.24**).

The enzymatic activity of crude native ArcA was also determined from 100 µl of lysate samples (5×10^7 CFU). The activities of crude native *SS2* ArcA were 0.21 and 0.20 U, respectively after 10 mM L-arginine inductions for 4 and 6 h. These activities were two times higher than that obtained from the HE06 *SS2* lysates cultured in the non-inducing conditions (lacking L-arginine). The crude native *SS2* ArcA was then investigated further for their physical properties, enzyme kinetics and inhibitory activities.

5.5.2 Determination of physical properties of crude native ArcA from *SS2*

The optimal temperature for crude native *SS2* ArcA activity was determined by conducting the ArcA activity assay (**Section 4.11.2**) at seven different points of temperature similar to that of previously described for the r*SS2* ArcA (**Section 5.4.7.1**). The results showed that, similar to *SS2* ArcA, the optimum temperature yielding the highest activity of crude native *SS2* ArcA was 42 ºC (**Table 5.17** and **Figure 5.25**). Moreover, unlike r*SS2* ArcA the temperature range for significant enzymatic activity of crude native *SS2* ArcA was extended up to 50 ºC (**Figures 5.25** and **5.18**).

An optimal pH for crude native *SS2* ArcA activity was determined by conducting the ArcA activity assay (**Section 4.11.2**) in different pH of assay buffer similar to that of previously described for the r*SS2* ArcA (**Section 5.4.7.2**). As shown in **Table 5.18** and **Figure 5.26**, the optimal pH for crude native *SS2* ArcA activity was in neutral or slightly acidic condition similar to that of obtained for the r*SS2* ArcA (**Section 5.3.7.2**).

Thermostability of crude native *SS2* ArcA was performed as previously described for the r*SS2* ArcA (**Section 5.4.7.3**). The results shown in **Table5.19** and **Figure 5.27** indicated that the activity of crude native *SS2* ArcA was stable at 4, 25 and 30 ºC for 3 h. However, similar to the activity of r*SS2* ArcA, the results also indicated that crude native *SS2* ArcA was thermosensitive when pre-

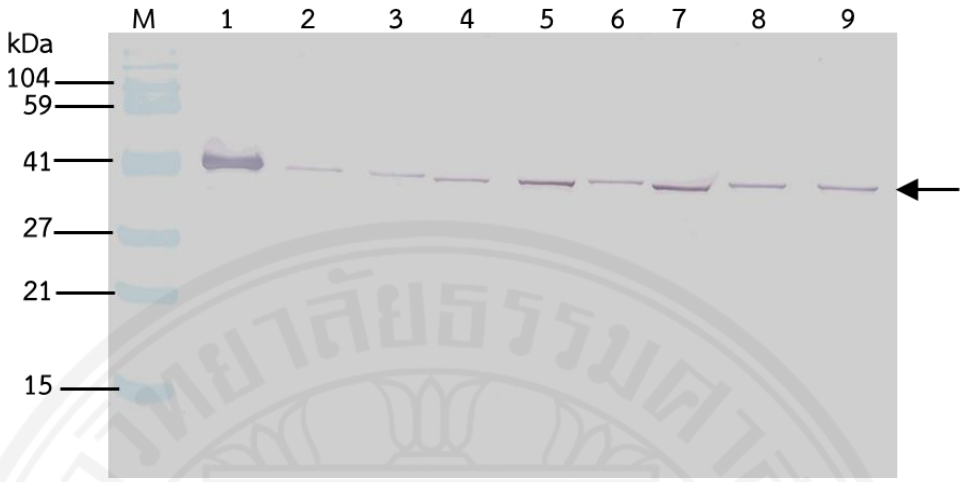

Figure 5.24 Western blot analysis of crude native *SS2* ArcA expression in HE06 *SS2* that were grown in low glucose, DMEM with lacking L-arginine or supplemented with 10 mM L-arginine at 37 ºC, in the presence of 5% CO_2 for 2, 4, 6 and 18 h

Lane M, Pre-stained broad range protein standard

Lane 1, Purified r*SS2* ArcA

Lanes 2, 4, 6, 8 Lysate of HE06 *SS2* grown in L-arginine depleted DMEM at 2, 4, 6 and 18 h, respectively

Lanes 3, 5, 7, 9 Lysate of HE06 *SS2* grown in L-arginine supplemented DMEM at 2, 4, 6 and 18 h, respectively

The expected mobility of the crude native *SS2* ArcA with size of 47-kDa is indicated by arrow. Molecular weight of protein markers are indicated as numbers shown on the left of figure

Table 5.17 Determination of optimal temperature for crude native *SS2* ArcA activity

Temperature (°C)	Mean of enzymatic activity±SD (U)
25	0.117±0.003
30	0.212±0.005
37	0.350±0.002
42	0.398±0.019
50	0.353±0.012
60	0.066±0.006
70	0.052±0.010

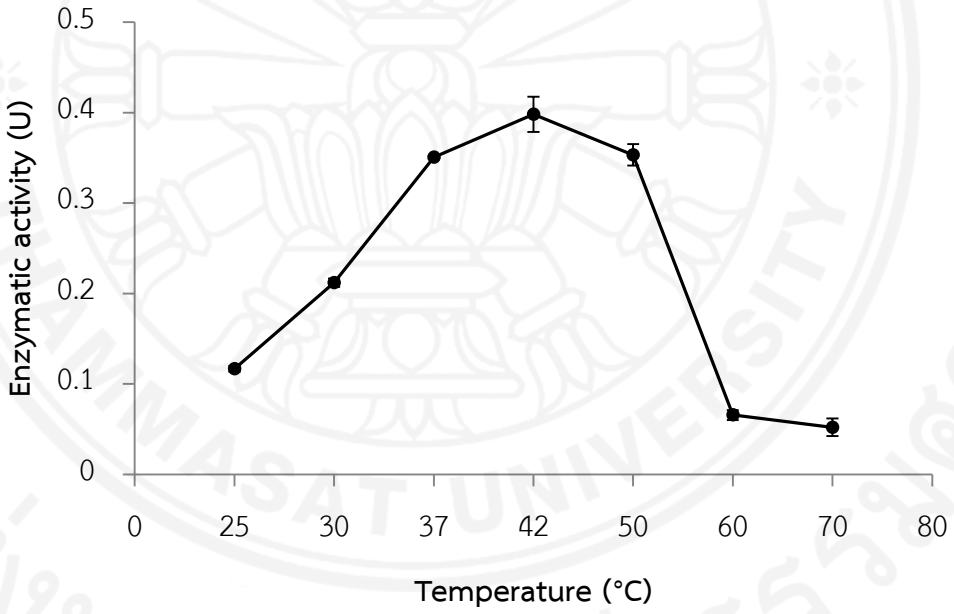

Figure 5.25 Temperature dependency of crude native *SS2* ArcA activity. The results are mean values obtained from three independent experiments with error bars indicating ± SD values

Table 5.18 Determination of optimal pH for crude native *SS2* ArcA activity

pH	Mean of enzymatic activity±SD (U)
4.2	0.167±0.016
4.5	0.198±0.006
5.0	0.215±0.003
5.5	0.247±0.018
6.0	0.270±0.016
6.2	0.283±0.014
6.5	0.317±0.001
7.2	0.351±0.007
7.5	0.246±0.013
8.0	0.031±0.002

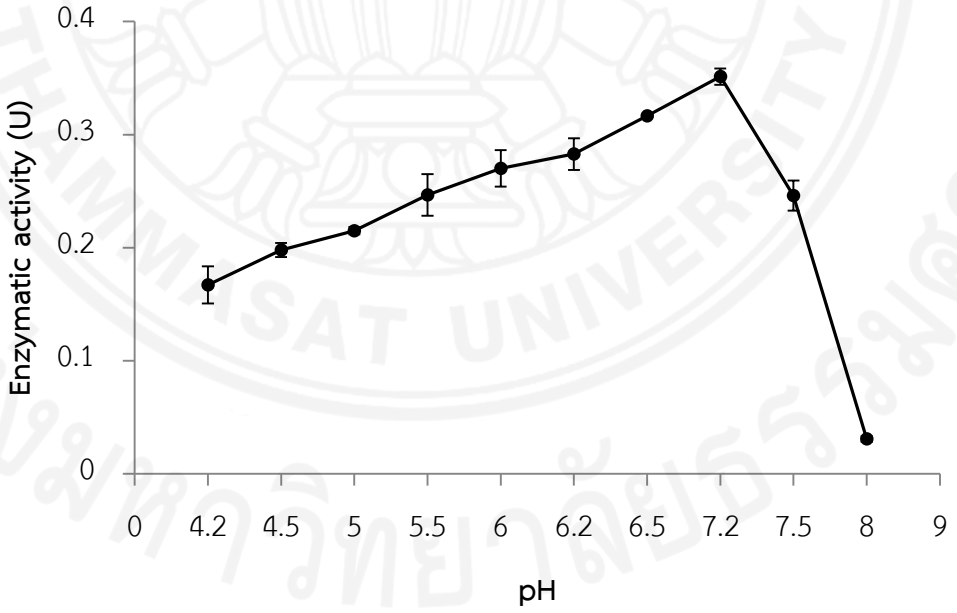

Figure 5.26 pH dependency of crude native *SS2* ArcA activity. The results are mean values obtained from three independent experiments with error bars indicating ± SD values

Table 5.19 Results of temperature stability of crude native *SS2* ArcA

Temperature (°C)	Mean of enzymatic activity±SD (U) at indicated pre-incubation time						
	0 min	30 min	60 min	90 min	120 min	150 min	180
4	0.297± 0.002	0.289± 0.002	0.290± 0.002	0.286± 0.001	0.280± 0.004	0.292± 0.002	0.288± 0.001
25	0.289± 0.007	0.291± 0.004	0.281± 0.005	0.286± 0.005	0.287± 0.007	0.283± 0.007	0.277± 0.001
30	0.290± 0.006	0.281± 0.006	0.282± 0.001	0.274± 0.002	0.271± 0.002	0.262± 0.001	0.248± 0.002
37	0.285± 0.001	0.277± 0.006	0.266± 0.003	0.208± 0.001	0.170± 0.001	0.141± 0.003	0.107± 0.005
42	0.283± 0.001	0.182± 0.003	0.086± 0.002	0.082± 0.001	0.061± 0.009	0.057± 0.005	0.037± 0.031

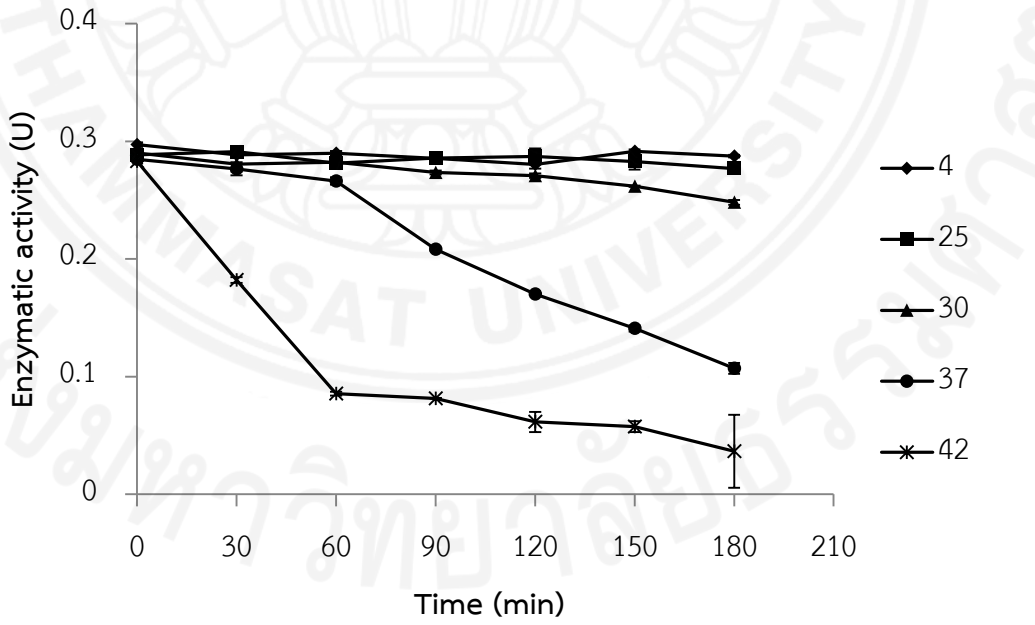

Figure 5.27 Temperature stability of crude native *SS2* ArcA. The enzyme solution was pre-incubated at 4 ºC (opened circle), 25 ºC (square), 30 ºC (triangle), 37 ºC (filled circle) and 42 ºC (star) for 3 h (kept it every 30 min for enzymatic assay). The results are mean values obtained from three independent experiments with error bars indicating ± SD values

incubation without L-arginine substrate at 37 ºC or 42 ºC though, the optimum temperature of them revealed at 42 ºC (**Section 5.4.7.1**).

The pH stability of of crude native *SS2* ArcA was performed as previously described for the r*SS2* ArcA (**Section 5.4.7.4**). As shown in **Table 5.20** and **Figure 5.28**, similar to r*SS2* ArcA, the crude native *SS2* ArcA activity was stable in pH ranging from 4.2 to 6.0 at 25 and 30 ºC for 3 h. Moreover, unlike r*SS2* ArcA, its activity appeared to remain fully stable under pH 4.2 to 5.0 at 37 ºC for 3 h.

The enzymatic properties of crude native *SS2* ArcA, K_m and V_{max} were determined as previously described for r*SS2* ArcA (**Section 5.4.8**). v_i of crude native *SS2* ArcA at a particular substrate concentration was measured and plotted against the substrate concentration to generate Michaelis-Menten plots. (**Table 5.21** and **Figure 5.29**). By using non-linear regression analysis, at 42 ºC the K_m and V_{max} values of crude native *SS2* ArcA were calculated as 0.157 mM and 0.149 U, respectively. The K_m and V_{max} values of crude native *SS2* ArcA at 37 ºC were 0.176 mM and 0.094 U, respectively (**Figure 5.29**).

5.5.3 Crude native *SS2* ArcA inhibition assay

The inhibition assay of crude native *SS2* ArcA was performed with 20 mM L-canavanine and 0.5 mM L-NIO and 0.08 U of crude native *SS2* ArcA (referring to V_{max} value in **Section 5.5.2**). The result showed that the crude native *SS2* ArcA was 60.53 % inhibited by 20 mM of L-canavanine and 56.58% inhibition by 0.5 mM L-NIO as shown in **Table 5.22**. Similar to r*SS2* ArcA, K_i of crude native *SS2* ArcA also could not be determined due to the lack of 100% enzyme inhibition as a reference point. As a consequence of these inhibitors demonstrated a partial inhibition for crude native *SS2* ArcA enzymatic activity, they were subsequently used to elucidate the role of *SS2* ArcA in facilitating HE06 *S. suis* survival under acidic environments.

Table 5.20 Result of pH stability of crude native *SS2* ArcA

Pre-incubation pH	Mean of enzymatic activity±SD (U) following 3 h pre-incubation		
	25 °C	30 °C	37 °C
4.2	0.289±0.003	0.276±0.008	0.258±0.003
5.0	0.283±0.008	0.271±0.010	0.257±0.007
6.0	0.272±0.000	0.263±0.010	0.194±0.011
7.2	0.195±0.016	0.188±0.015	0.149±0.009
8.0	0.176±0.012	0.172±0.019	0.115±0.016

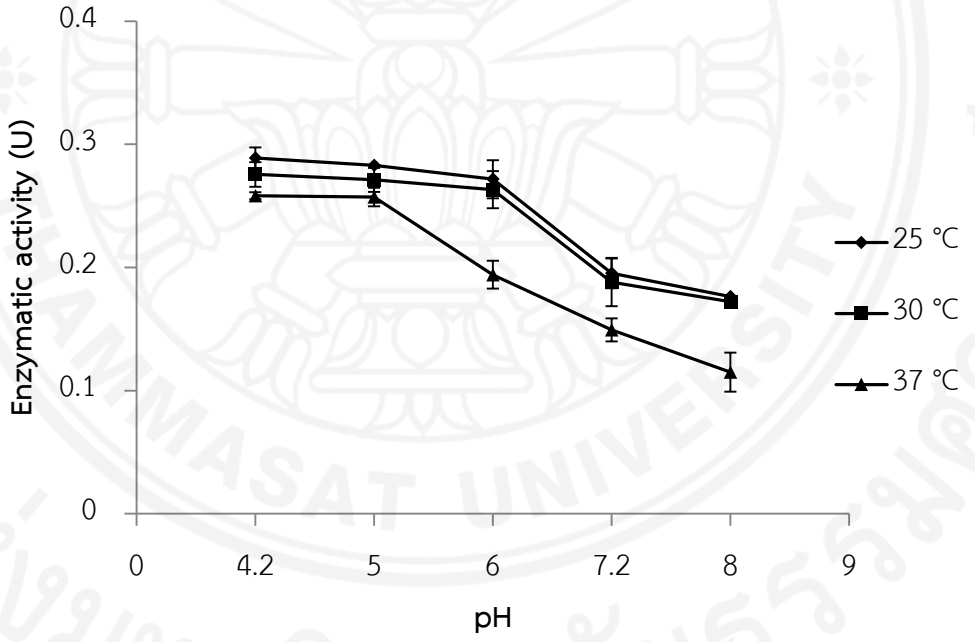

Figure 5.28 The pH stability of crude native *SS2* ArcA was determined by pre-incubating crude native ArcA enzyme in potassium phosphate buffer at different pH (assay buffer pH 4.2, 5.0, 6.0, 7.2 and 8.0, respectively). These reactions were conducted at 25 (square), 30 (circle) and 37 °C (triangle) for 3 h and followed by *SS2* ArcA assay. The results are mean values obtained from three independent experiments with error bars indicating ± SD values

Table 5.21 Enzymatic activity of each concentration of L-arginine substrate for determining K_m and V_{max} values of crude native *SS2* ArcA

L-arginine	Mean of enzymatic activity±SD (U)	
(mM)	42 °C	37 °C
8	0.147±0.001	0.094±0.002
4	0.143±0.001	0.093±0.003
2	0.140±0.007	0.089±0.004
1	0.126±0.004	0.076±0.003
0.5	0.113±0.004	0.061±0.001
0.25	0.092±0.003	0.054±0.001
0.125	0.063±0.003	0.047±0.001
0.063	0.042±0.003	0.031±0.002
0.031	0.032±0.004	0.010±0.001
0.016	0.018±0.002	0.008±0.009
0.008	0.015±0.001	0.004±0.007

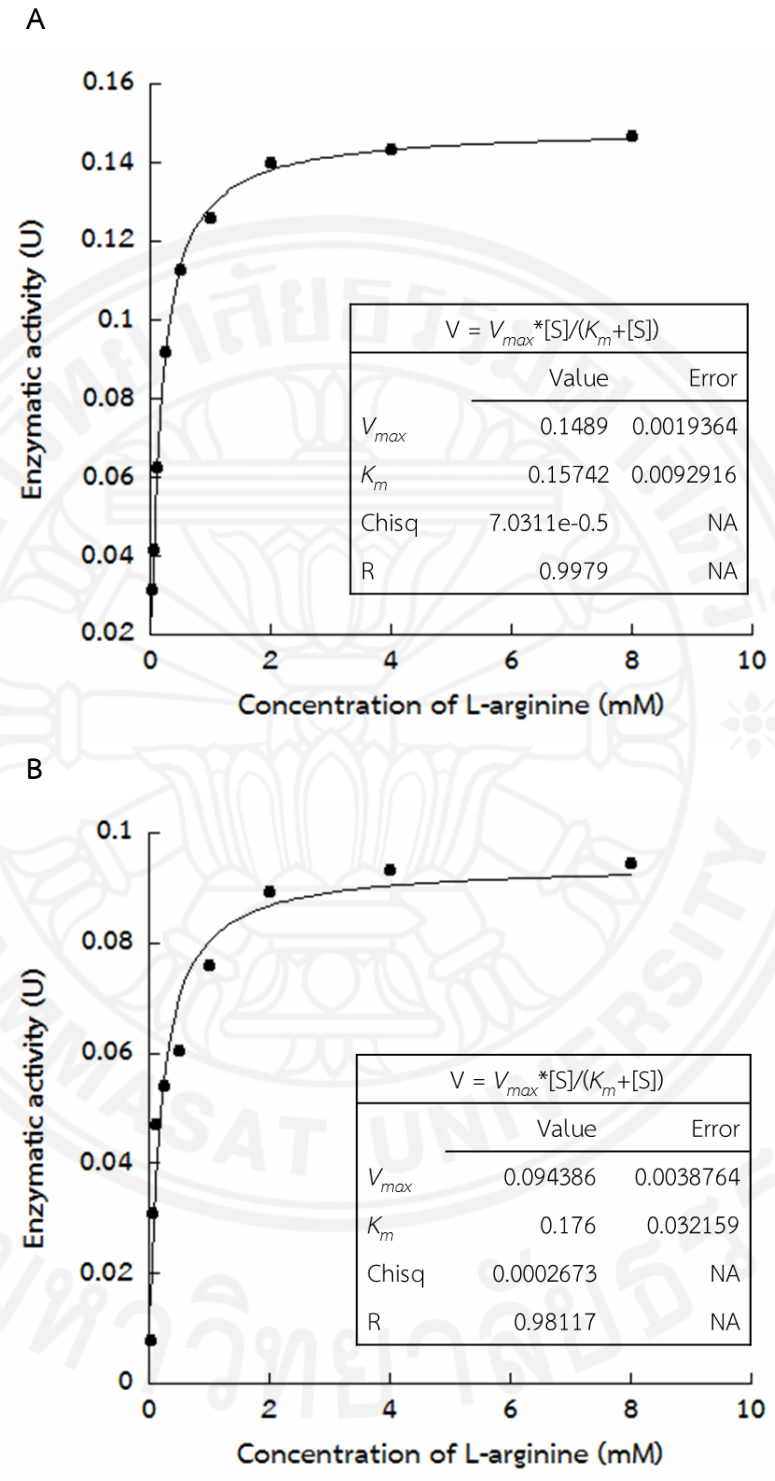

Figure 5.29 Michaelis-Menten Plot. L-arginine substrate concentrations *versus* enzymatic activity of crude native *SS2* ArcA. K_m and V_{max} as shown in this plot (A) Assay was conducted at 42 ºC and (B) Assay was conducted at 37 ºC. The results are mean values obtained from triplicate independent experiments

Table 5.22 Inhibition assay of crude native ArcA in *SS2*

Reaction mixture	Enzymatic activity (U)	Inhibition (%)
Crude native *SS2* ArcA original activity		
Crude native *SS2* ArcA + 10 mM L-arginine	0.077±0.021	–
Negative inhibition control		
Crude native *SS2* ArcA + UDW + 10 mM L-arginine	0.076±0.009	–
L-canavanine inhibitory test		
Crude native *SS2* ArcA + 20mM L-canavanine + 10 mM L-arginine	0.030±0.007	60.53
L-NIO inhibitory test		
Crude native *SS2* ArcA + 0.5 mM L-NIO + 10 mM L-arginine	0.033±0.027	56.58

5.6 Assessment of the role of *SS2* ArcA in facilitating HE06 *SS2* survival under acidic environments.

5.6.1 Expression and enzymatic activity of *SS2* ArcA in HE06 *SS2* grown under the acidic environment

To assess the expression of *SS2* ArcA in *S. suis* cultured in acidified media, HE06 *SS2* were grown in DMEM without L-arginine (Sigma) at 37 ºC in 5% CO_2/95% air at pH of 4.0, 6.0 and 7.5, respectively. The 10 mM L-arginine was added to induce *SS2* ArcA expression and the cultures were incubated for 4 and 6 h. Approximately 5 × 10^8 CFU/ml bacterial cells were harvested and resuspended in assay buffer to prepared whole cell lysate. Bacterial lysate (10 µl) were mixed with

sample buffer and analyzed by Western blot probed with mouse anti-r*SS2* ArcA polyclonal antibody. Western blot results revealed that the *SS2* ArcA expression in HE06 *SS2* was gradually increased depending on the incubation time in all tested pH. However, there is no obvious difference in ArcA expression observed in HE06 *SS2* lysate prepared from bacteria grown in either L-arginine depleted or supplemented DMEM medium in all tested pH (**Figure 5.30**). Moreover, these data suggested that culture of HE06 *SS2* in acidified medium (pH 4.0 and 6.0) did not revealed any change in *SS2* ArcA expression compared to that of the neutral medium (pH 7.5) (**Figure 5.30**).

In order to quantify the enzymatic activity of the mentioned above bacterial lysates, the cell lysate (100 µl; 5×10^7 CFU) was subjected to ArcA activity assay as decribed in **Section 4.11.2**. It was found that HE06 grown in neutral pH DMEM supplemented with L-arginine provided ArcA activity approximately two times higher than that of grown in medium without L-arginine. While ArcA activity of HE06 grown in acidified DMEM (pH 6.0 and 4.0) supplemented with L-arginine was approximately three times higher than that of HE06 grown in the acidified medium without L-arginine (**Figure 5.31**). Collectively, these data indicated that although, L-arginine induction of ArcA expression in HE06 *SS2* grown in acidified medium did not apparently induce higher amount of ArcA, it induced higher activity of ArcA.

5.6.2 Attempted construction of *arcA* mutant of HE06 *SS2*

Initially, to facilitate analysis of the contribution of *SS2 arcA* in mediating the survival of *SS2* under acidic environments, an *arcA*-negative derivative of HE06 *SS2* was constructed.

5.6.2.1 Attempted construction of *arcA* mutant of HE06 *SS2* by using insertion duplication mutagenesis

To generate *arcA* mutant of HE06 *SS2*, the 814-bp *arcA* fragment of HE06 *SS2* was amplified using primers *arcA*-A F and R3 reverse which incorporated *Bam*HI site (**Table 4.4**). The 814-bp *arcA* amplicon was then digested with *Bam*HI to generate a 323-bp *Bam*HI-*arcA* fragment containing one out of the four conserved motifs of the guanidine-group-modifying enzyme involving in L-arginine substrate binding (**Figure 5.32A**). The purified 323-bp *Bam*HI-*arcA* fragment

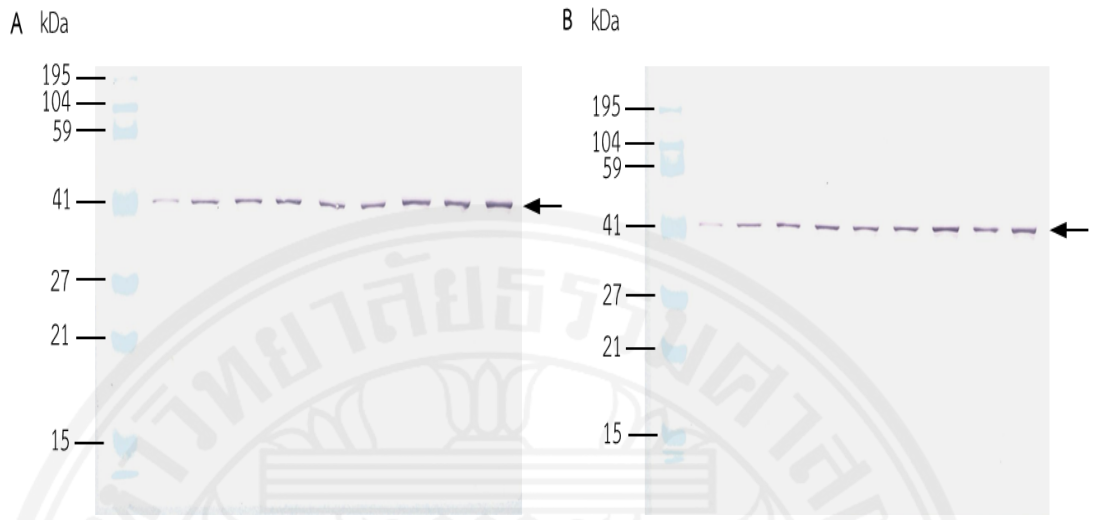

Figure 5.30 Western blot analysis of *SS2* ArcA expression in HE06 *SS2* grown in acidified medium

Panel A Lane M, Pre-stained broad range protein standard
Lane 1, lysate of HE06 inoculum
Lane 2–3, lysate of HE06 grown in L-arginine depleted and supplemented DMEM pH 7.5 for 4 h
Lane 4–5, lysate of HE06 grown in L-arginine depleted and supplemented DMEM pH 7.5 for 6 h
Lane 6–7, lysate of HE06 grown in L-arginine depleted and supplemented DMEM pH 6.0 for 4 h
Lane 8–9, lysate of HE06 grown in L-arginine depleted and supplemented DMEM pH 6.0 for 6 h

Panel B Lane M and 1–5 were similar to that of presented in Panel A
Lane 6–7, lysate of HE06 grown in L-arginine depleted and supplemented DMEM pH 4.0 for 4 h
Lane 8–9, lysate of HE06 grown in L-arginine depleted and supplemented DMEM pH 4.0 for 6 h

The expected mobility of the crude native *SS2* ArcA with size of 47-kDa is indicated by arrow. Molecular weight of protein markers are indicated as numbers shown on the left of figure

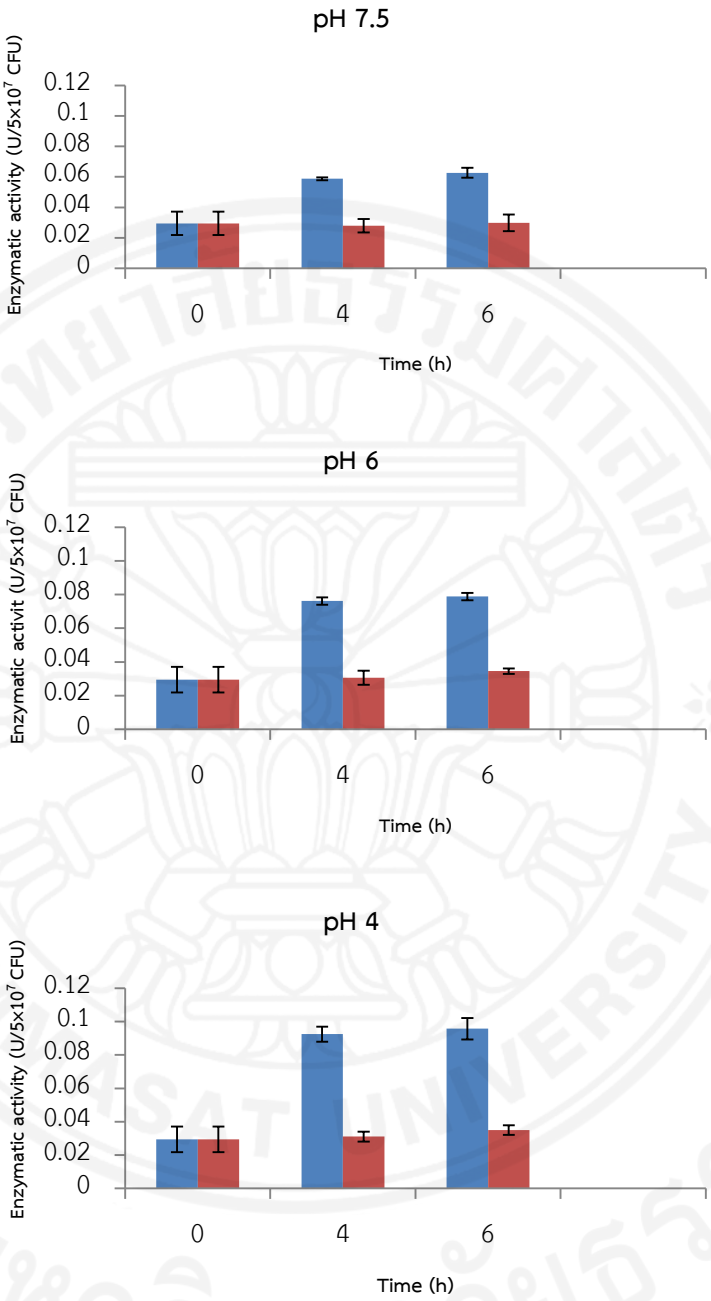

Figure 5.31 ArcA activity of HE06 SS2 grown in neutral and acidified DMEM lacking or supplementing with L-arginine (pH 4.0, 6.0 and 7.5). The results are expressed as mean values obtained from three independent experiments with error bars indicating ± SD values. Blue bars represent ArcA activities from HE06 lysate grown in DMEM supplemented with L-arginine and red bars represent ArcA activities from HE06 lysate grown in DMEM without L-arginine

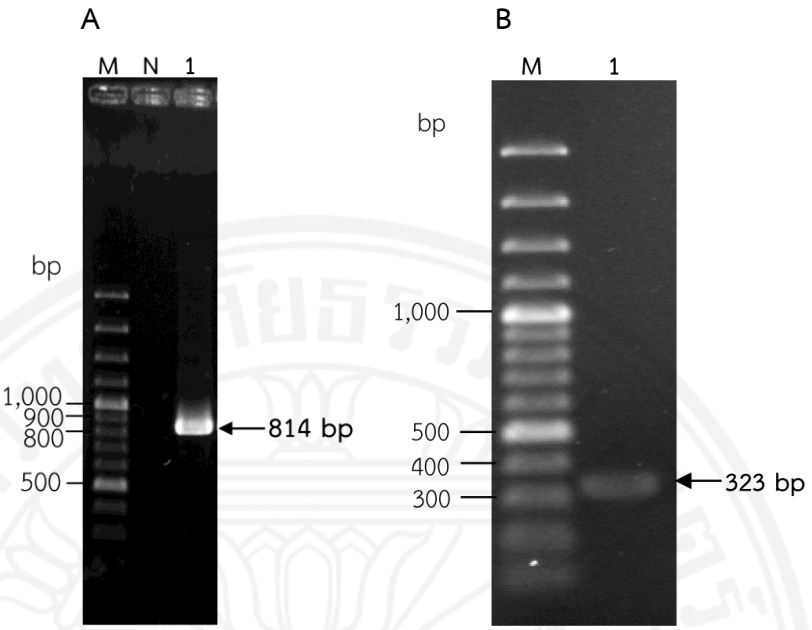

Figure 5.32 Cloning of *arcA* gene into pVA891

Panel A PCR amplicon of 814-bp ArcA coding sequence

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane 1, 814-bp *SS2 arcA* amplicon is indicated by arrow

Panel B *Bam*HI digestion of 814-bp *arcA* amplicon

Lane M, GeneRuler™ 1 kb DNA ladder

Lane 1, Purified 323-bp *arcA* following the *Bam*HI-digestion of 814-bp *arcA* amplicon (arrow)

Numbers on the left are the mobility of DNA size markers in bp

was ligated into the *Bam*HI digested plasmid pVA891 to generate pVA891*::*323-bp *arcA* (**Sections 4.12.1.1** and **4.12.1.2**, **Figure 5.32B**). This construct was then transformed into competent DH5α *E. coli* cells and selected by plating on LB agar supplemented with 25 µg/ml chloramphenicol and 125 µg/ml erythromycin (**Sections 4.9.4** and **4.9.5, Appendix A**). Insertion of 323-bp *arcA* into pVA891 vector (designated pVA891*::*323-bp *arcA*) was screened by colony PCR using F pVA_Cm and R3 reverse primers (**Table 4.4**) and *Bam*HI restriction enzyme digestion (**Figures 5.33A** and **5.33B**). *E. coli* transformant clone number 3 carrying 323-bp *arcA* insert

was selected for further studies. The recombinant plasmid was then extracted, purified, and electroporated into the HE06 *SS2* (**Sections 4.9.6.1**, **4.9.6.2**, **4.12.1.4** and **4.12.1.5**). The erythromycin-resistant colonies were selected and screened by colony PCR using (*arcA*-A F and *arcA*-A R) and (*arcA*-A F and R3 reverse) (**Table 4.4**) for the absent of 814 and 1,227-bp PCR product. The construct strategy is illustrated in **Figure 5.34**. Despite, several attempts were made to electroporate the recombinant shuttle plasmid pVA891*::*323-bp *arcA* into HE06 *SS2* competent cells, none of these were successful (**Figure 5.35**).

5.6.2.2 Attempted construction of *arcA* mutant of HE06 *SS2* by using shuttle thermosensitive suicide vector based on allelic exchange mutagenesis

In view of lacking success in mutagenesis using insertion duplication, the attempts were made to construct *arcA* mutant using allelic exchange mutagenesis. The major portion of *SS2 arcA* was deleted and replaced with a *gfp* reporter using the shuttle thermosensitive suicide vector pSET4S.[132] The entire *arcA* gene with additional 2,000-bp flanking regions (3,227-bp) was amplified from HE06 *SS2* genomic DNA using oligonucleotide primers Forward ups and Reverse dns (**Table 4.4** and **Section 4.12.2.1**). The 3,227-bp amplicon (**Figure 5.36A**) was purified and ligated into the T-overhang of the pGEM-T cloning vector (**Section 4.9.3** and **Table 4.3**) to generate pGEM-T*::*3,227-bp. This construct was then transformed into competent DH5α *E. coli* cells (**Sections 4.9.4** and **4.9.5**). Insertion of 3,227-bp amplicon into pGEM-T cloning vector was screen by colony PCR using two primer-pairs (Forward ups/Reverse dns and *arcA*-B F/ *arcA*-B R) (**Table 4.4**). The PCR products 3,227-bp and 403-bp were obtained from two out of the three randomly selected colonies (**Figure 5.36B**). *E. coli* transformant clone number 9 carrying the 3,227-bp insert was selected for further studies.

The recombinant plasmid from clone pGEM-T*::*3,227-bp number 9 was extracted (**Section 4.9.6.2**) and subjected to DNA sequencing analysis. BLASTN analysis indicated that the nucleotide sequence of 3,227 bp derived from HE06 *SS2* was identical to 3,227-bp sequence of the reference *SS2* strain P1/7 in the Genbank database (accession number AM946016.1). Subsequently, plasmid DNA of

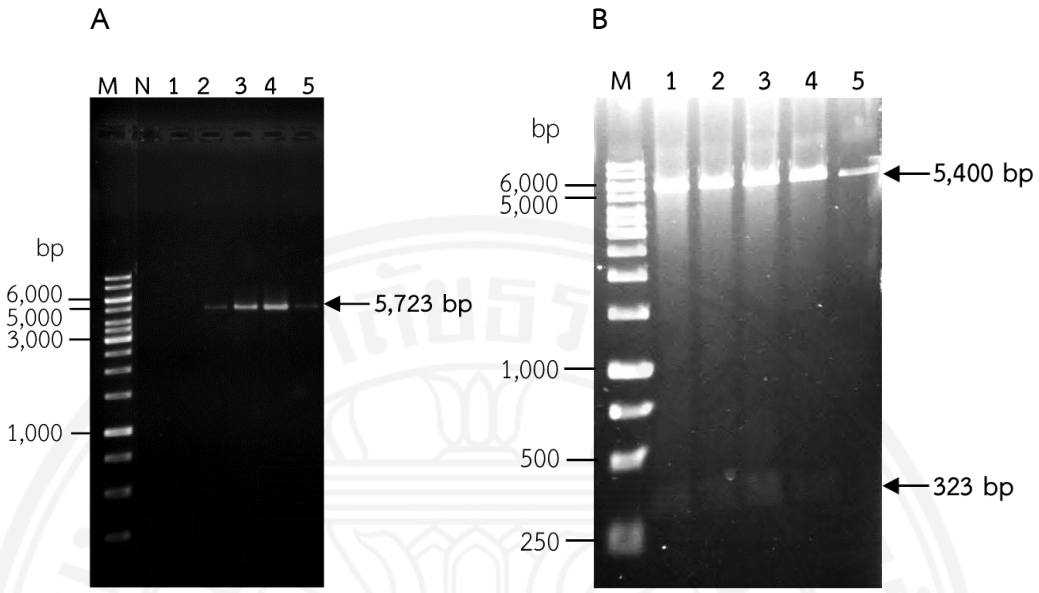

Figure 5.33 Screening of DH5α *E. coli* carrying pVA891*::*323-bp *arcA*

Panel A PCR screening of four randomly selected colonies of pVA891*::*323-bp transformed DH5α *E. coli*

Lane M, GeneRuler™ 1 kb DNA ladder

Lane N, No template control

Lane 1, Empty vector

Lane 1–4, PCR amplicon of four randomly selected colonies of pVA891*::*323-bp *arcA* transformed DH5α *E. coli* clones

The 5,723-bp of pVA891*::*323-bp *arcA* amplicons is indicated by arrow.

Panel B *Bam*HI restriction endonuclease digestion of pVA*::*323-bp *arcA*. Following the digestion, the digested DNA mixtures were analyzed by 0.8% TBE-agarose gel electrophoresis

Lane M, GeneRuler™ 1 kb DNA ladder

Lane 1–4, *Bam*HI digested pVA891*::*323-bp *arcA* from recombinant clones no. 1-4

Lane 5, *Bam*HI digested pVA891

The *Bam*HI-digested pVA*::*323-bp *arcA*, *arcA* (323 bp) and pVA891 (5,400 bp) DNA bands are indicated by arrows.

Numbers on the left are the mobility of DNA size markers in bp

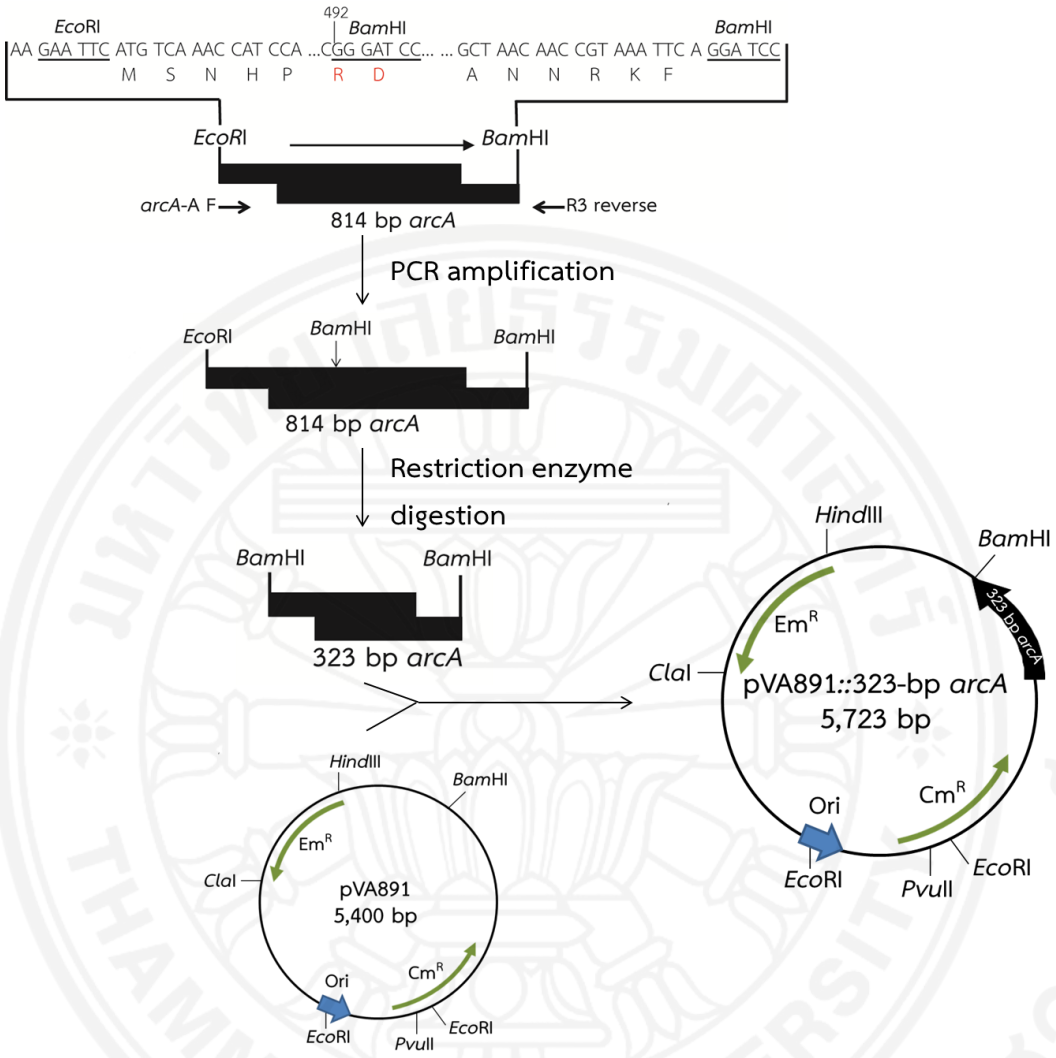

Figure 5.34 Construction of pVA891::323-bp *arcA*. A *SS2 arcA* DNA fragment (814-bp) was amplified by PCR using oligonucleotides primer *arcA*-A F (*Not*I) and R3 reverse (*Bam*HI). The PCR product was digested with *Bam*HI. The 323-bp *Bam*HI fragment was then ligated into the *Bam*HI digested shuttle suicide vector pVA891 in order to construct plasmid pVA891::323-bp *arcA*. This plasmid was transformed into the DH5α *E. coli*. The black block-arrow indicates the DNA region of the *SS2 arcA* open reading frame (ORF). The oligonucleotides used are designated by the small thick arrows accompanied by the primer names. The horizontal thin arrow indicates the direction of transcription of the *SS2 arcA* ORF in PCR amplification product. The *SS2* ArcA DNA and deduced amino acid sequences are shown by black and the residues that involve with substrate binding site are indicated by red. The plasmid maps are not to scale

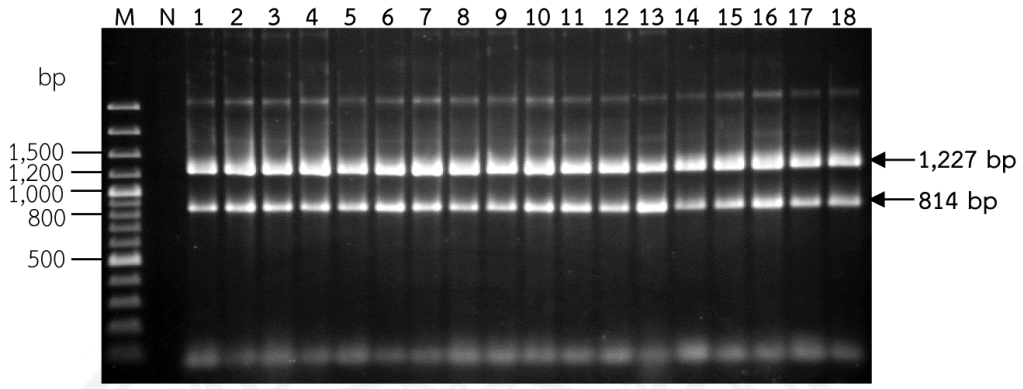

Figure 5.35 PCR screening of 17 randomly selected colonies of pVA891*::*323-bp *arcA* transformed HE06 *SS2*

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane N, Negative control (no template)

Lane 1, PCR amplicon of genomic DNA from HE06 *SS2*

Lane 2-18, PCR amplicon of 17 randomly selected colonies of pVA891*::*323-bp *arcA* transformed HE06 *SS2*

The expected sizes of PCR amplicons are indicated by arrows.

Numbers on the left are the mobility of DNA size markers in bp

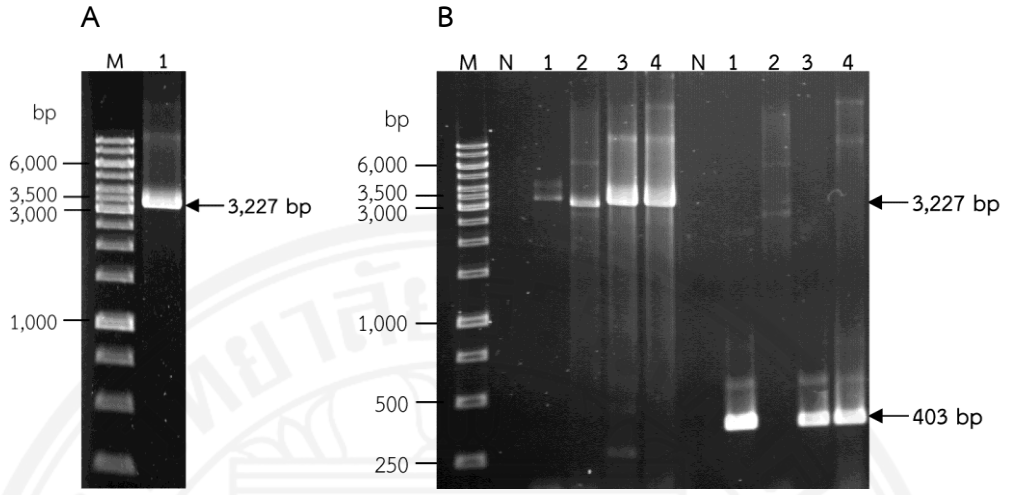

Figure 5.36 PCR amplicon of 3,227-bp *arcA* and PCR screening for pGEM-T::3,227-bp transformed DH5α *E. coli* clones

Panel A PCR amplicon of 3,227-bp *arcA* coding sequence and flanking regions

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane 1, PCR amplicon of 3,227-bp coding sequence

Panel B PCR screening for pGEM-T::3,227-bp transformed DH5α *E. coli* clones.

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane N, Negative control (no template)

Lane 1, PCR amplicons from genomic DNA in HE06 *SS2*

Lane 2-4, PCR amplicon of 3 randomly selected colonies (no. 1, 5 and 9) of pGEM-T::3,227-bp transformed DH5α *E. coli* clones

The expected size of pGEM-T::3,227-bp (3,227 bp) and *arcA* as internal fragment (403 bp) amplicons are indicated by arrows.

Numbers on the left are the mobility of DNA size markers in bp

pGEM-T::3,227-bp was used as template in an inverse PCR using primers FC inverse and RC inverse which were incorporated with *Nhe*I (**Table 4.4**). An expected 5,015-bp Inverse PCR amplicon (**Figure 5.37A**) was then excised and purified from agarose gel (**Section 4.9.2** and **Figure 5.37B**). The purified amplicon was then digested with *Nhe*I, and re-circularized. Subsequently, the plasmid was subjected to PCR for amplifying the 2,000 bp flanking region using primers Forward ups and Reverse dns (**Table 4.4**).

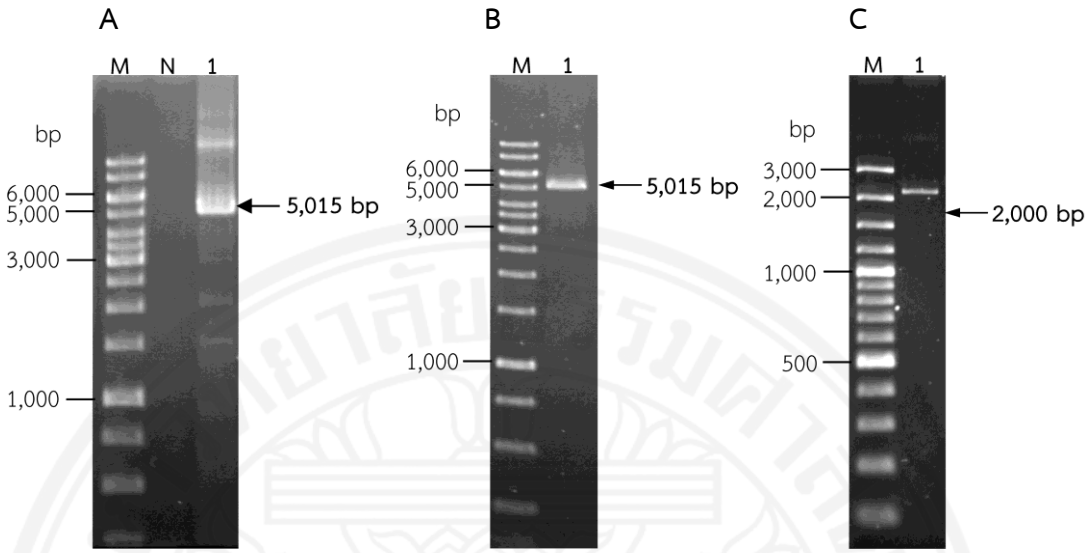

Figure 5.37 5,015-bp of inverse PCR amplicon and PCR amplicon of 2,000-bp flanking regions of *arcA*

Panel A The 5,015-bp of inverse PCR amplicon
Lane M, GeneRuler™ 100 bp plus DNA ladder
Lane 1, PCR amplicon of 5,015-bp is indicated by arrow

Panel B Purified 5,015-bp inverse PCR amplicon
Lane M, GeneRuler™ 100 bp plus DNA ladder
Lane 1, Purified PCR amplicon of 5,015-bp is indicated by arrow

Panel C PCR amplicon of 2,000-bp flanking regions of *arcA*
Lane M, GeneRuler™ 100 bp plus DNA ladder
Lane 1, PCR amplicon of 2,000-bp flanking regions of *arcA*
Numbers on the left are the mobility of DNA size markers in bp

The purified 2,000-bp PCR product was ligated into the T-overhang of the pGEMT cloning vector (**Section 4.9.3** and **Figure 5.37C**) to generate pGEMT::2,000-bp. This construct was then transformed into competent DH5α *E. coli* cells (**Sections 4.9.4** and **4.9.5**), The 2,000-bp insertion of amplicon into pGEM-T cloning vector (designated pGEM-T::*ΔarcA*) was screened by colony PCR using primers (M13F(-40) and M13R(-40)), (Forward ups and Reverse dns), and (*arcA*-B F and *arcA*-B R) (**Table 4.4**). The PCR product of 2,250-bp was obtained using (M13F(-40) and M13R(-40)

(including 2,000-bp amplicon and vector multi-cloning sites flanking regions) (**Figure 5.38A**). In addition, 1,227-bp and 403-bp of PCR products were absence from pGEM-T*::ΔarcA* the as shown in **Figure 5.38B**, Lane number 3. The expected transformant clone was clone number 16 (**Figure 5.38**). *E. coli* transformant clone number 16 carrying the 2,000 bp flanking regions of *arcA* was then selected for further studies. The pGEM-T*::ΔarcA* recombinant plasmid was extracted and used as template for amplifying 2,000-bp fragment using *Pfu* DNA polymerase as shown in **Figure 5.39A**. This purified PCR product was subsequently blunted-end ligated into *Sma*I site of digested pSET4S vector (**Figure 5.39B**) and transformed into the competent DH5α *E. coli* (**Sections 4.9.4** and **4.9.5**). The spectinomycin-resistant colonies were selected and screened by PCR using F pSET4S and R pSET4S primers (**Table 4.4**) for the presence of expected 3,715-bp PCR product, containing 2,000-bp *arcA*-flanking region and pSET4S multi-cloning sites (1,715-bp) (designated pSET4S*::ΔarcA*) (**Figure 5.40**). The expected transformant clone was obtained from three out of the 33 randomly selected colonies. The recombinant plasmid from clone pSET4S*::ΔarcA* number 9 was extracted, purified and further digested with *Nhe*I. A *gfp* fragment was amplified from pGFP vector using *gfp* forward and *gfp* reverse primers which incorporated *Nhe*I sites (**Section 4.12.2.1**, **Table 4.4** and **Figure 5.41A**). Subsequently, purified *gfp* amplicon was digested and ligated into *Nhe*I-digested pSET4S*::*2,000-bp. The spectinomycin- resistant colonies were selected and screened by PCR using *gfp* forward and *gfp* reverse primers for the presence of 750-bp PCR product containing *gfp* gene (**Figure 5.41B**). *E. coli* transformant clone number 1 carrying the pSET4S*::*2,000-bp and *gfp* (designated pSET4S*::ΔarcA::gfp*) was selected for further studies (**Figure 5.42A**). The recombinant plasmid pSET4S*::ΔarcA::gfp* was extracted and eloctroporated into HE06 *SS2* (**Sections 4.12.2.2** and **4.12.2.3**). The spectinomycin-resistant colonies were selected and screened by PCR using *gfp* forward and *gfp* reverse primers. Nevertheless, several attempts were made to transformed the pSET4S*::ΔarcA-gfp* into HE06 *SS2* cells but none of these were successful (**Figure 5.42B**).

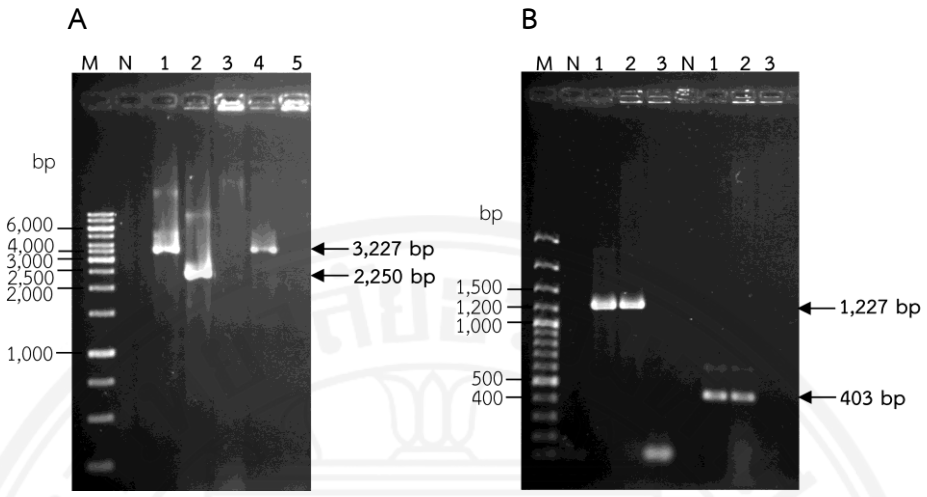

Figure 5.38 PCR screening and verifying of selected colonies of pGEM-T*::ΔarcA* transformed DH5α *E. coli* clones

Panel A PCR screening of four randomly selected colonies of pGEM-T*::ΔarcA* transformed DH5α *E. coli* clones

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane N, Negative control (no template)

Lane 1, PCR amplicon from genomic DNA in HE06 *SS2*

Lane 2-5, PCR amplicon of randomly selected colonies (no. 16-19) of pGEM-T*::ΔarcA* transformed DH5α *E. coli* clones

The expected sizes of pGEM-T*::ΔarcA* (2,250 bp) and *arcA* with flanking regions (3,227 bp) amplicons are indicated by arrows.

Panel B PCR verifying of selected colonies of pGEM-T*::ΔarcA* transformed DH5α *E. coli* clones

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane N, Negative control (no template)

Lane 1, PCR amplicon from genomic DNA in *SS2* HE06

Lane 2-3, PCR amplicons of clones no. 18 and 16 (Lane 4 and 2 in **Figure 5.38A**) of pGEM-T*::ΔarcA* transformed DH5α *E. coli* clones

The expected transformant clone of pGEM-T*::ΔarcA* did not have *arcA* amplicons (1,227 and 403 bp) as indicated by arrows.

Numbers on the left are the mobility of DNA size markers in bp

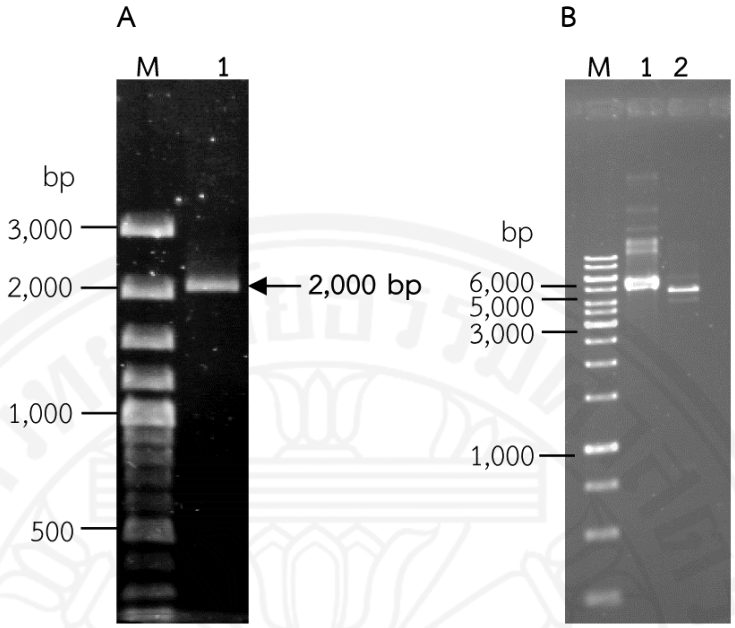

Figure 5.39 PCR amplicon of 2,000-bp flanking regions of *arcA* and *Sma*I-digested pSET4S vector

Panel A PCR amplicon of 2,000-bp flanking regions of *arcA* using pGEM-T::*ΔarcA* clone no. 16 as the DNA template

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane 1, PCR amplicon of 2,000-bp flanking regions of *arcA* (arrow)

Panel B *Sma*I-digested pSET4S vector

Lane M, GeneRuler™ 1 kb DNA ladder

Lane 1, undigested pSET4S vector

Lane 2, *Sma*I digested pSET4S vector

Numbers on the left are the mobility of DNA size markers in bp

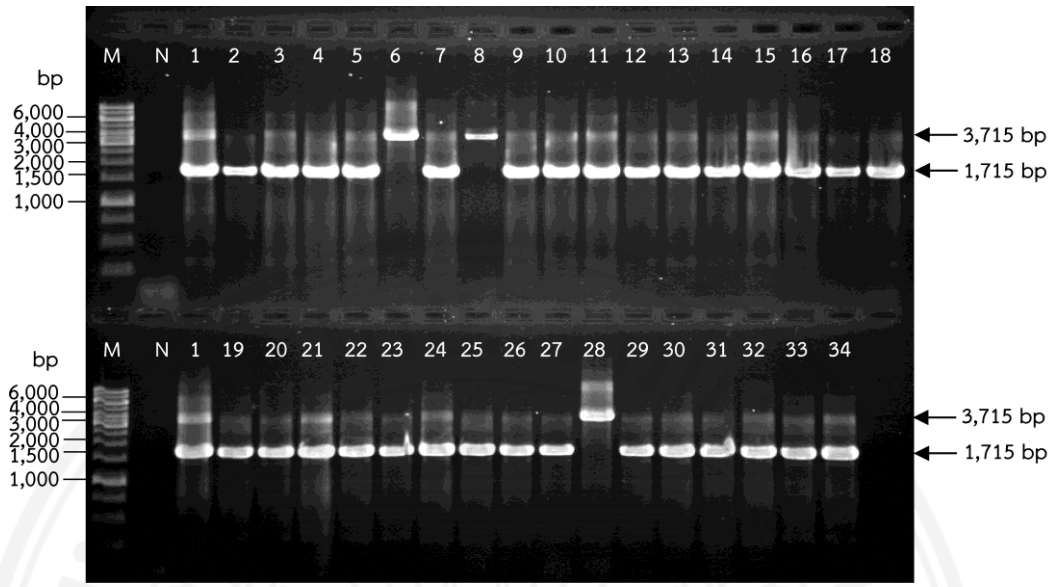

Figure 5.40 PCR screening of 33 randomly selected colonies of pSET4S*::ΔarcA* transformed DH5α *E. coli* clones

Lane M, GeneRuler™ 1 kb DNA ladder

Lane N, Negative control (no template)

Lane 1, PCR amplicons from pSET4S multi-cloning sites

Lane 2-34, PCR amplicons of randomly selected 33 colonies of pSET4S*::ΔarcA* transformed DH5α *E. coli* clones

The expected sizes of pSET4S*::ΔarcA* (3,715 bp) and pSET4S multi-cloning sites (1,715 bp) amplicons are indicated by arrows.

Numbers on the left are the mobility of DNA size markers in bp

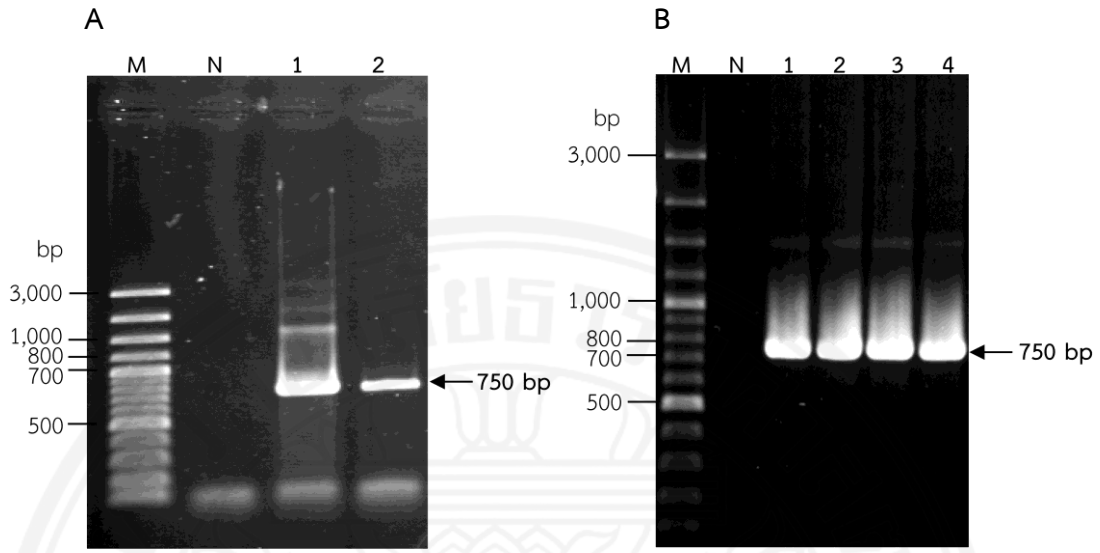

Figure 5.41 PCR amplicon of *gfp* gene

Panel A PCR amplicon of *gfp* gene (750 bp)

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane 1, PCR amplicon of *gfp* (750 bp) as indicated by arrow

Panel B PCR screening of pSET4S*::ΔarcA::gfp* transformed DH5α *E. coli* clones

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane N, Negative control (no template)

Lane 1, PCR amplicon of *gfp* (750 bp)

Lane 2-4, PCR amplicons of randomly selected colonies of pSET4s*::ΔarcA::gfp* transformed DH5α *E. coli* clones

The expected size of *gfp* (750 bp) amplicons is indicated by arrows

Numbers on the left are the mobility of DNA size markers in bp

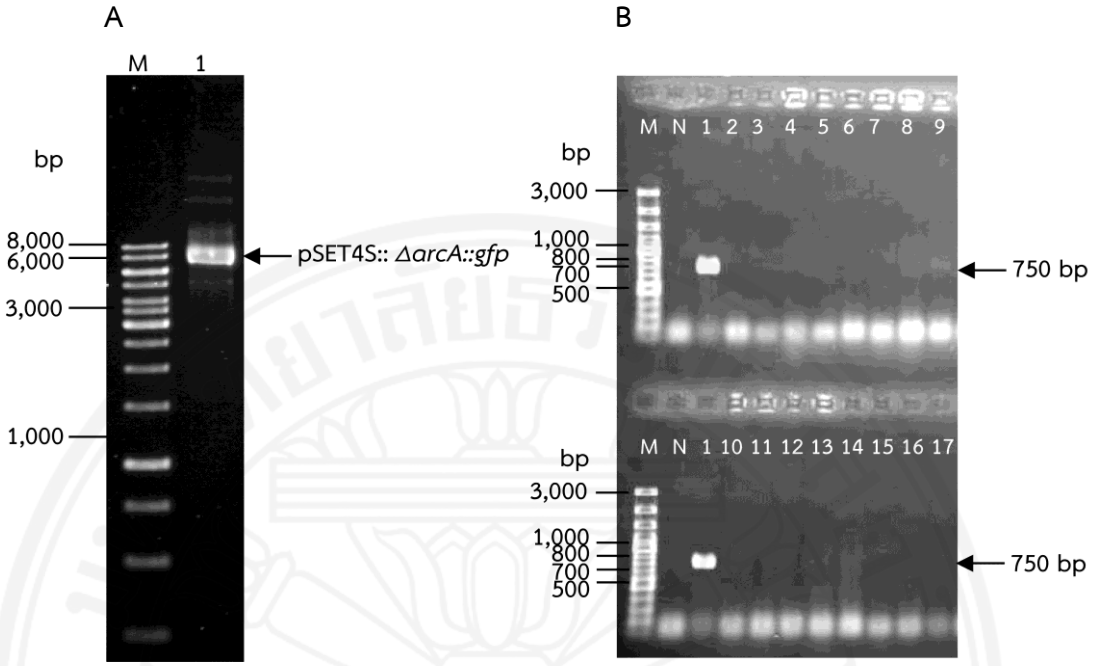

Figure 5.42 Plasmid extraction and PCR screening of selected colonies of pSET4S*::ΔarcA::gfp* transformed HE06 *SS2*

Panel A Plasmid extraction from recombinant clone pSET4S*::ΔarcA::gfp* no. 9

Lane M, GeneRuler™ 1 kb DNA ladder

Lane 1, 7,256 bp of pSET4S*::ΔarcA::gfp* is indicated by arrows

Panel B PCR screening of 16 randomly selected colonies of pSET4S*::ΔarcA::gfp* transformed HE06 *SS2*

Lane M, GeneRuler™ 100 bp plus DNA ladder

Lane N, Negative control (no template)

Lane 1, PCR amplicon of *gfp* (750 bp) using recombinant clone carrying pGFP vector as a DNA template

Lane 2-47, PCR amplicon of 16 randomly selected colonies of pSET4S*::ΔarcA::gfp* transformed HE06 *SS2*

The expected size of PCR amplicons is indicated by arrows

Numbers on the left are the mobility of DNA size markers in bp

5.6.3 The contribution of *SS2* ArcA in the survival of *S. suis* under acidic condition

As a consequence of unsuccessful in construction of *arcA*-negative derivative of HE06 *SS2*, the ability of ArcA to facilitate the survival of HE06 *SS2* was examined using ArcA enzyme inhibitors, L-canavanine and L-NIO. The HE06 *SS2* were grown and resuspended into approximately 1×10^7 CFU/ml in DMEM at pH 4.0, 6.0 and 7.5 respectively. The culture were then supplemented with 10 mM L-arginine and 20 mM L-canavanine (or 0.5 mM L-NIO) and incubated further for 4 and 6 h. The total numbers of HE06 *SS2* then were collected from each testing DMEM cultures and enumerated by plating on blood agar plate (**Section 4.11.6**). In both neutral and acidified DMEM supplemented with 10 mM L-arginine, numbers of HE06 showed approximately 10 to 50-fold increasing relative to the inoculum size ($p < 0.01$) (**Figure 5.43**). While in 20 mM L-canavanine and L-NIO supplemented DMEM, numbers of HE06 were remained relatively to that of the inoculum size following 4 h incubation. Interestingly, the numbers of HE06 were slightly increased in DMEM without L-arginine but numbers of HE06 in 20 mM L-canavanine supplementation still remained similar to the inoculum following the 6 h incubation (**Figure 5.43**). For the L-NIO supplemented DMEM pH 6.0 and 7.5, the numbers of HE06 became below the inoculum following the 6 h incubation ($p < 0.01$) (**Figure 5.43**). These data suggested that L-arginine and ArcA activity were required for HE06 *SS2* multiplication (**Section 5.5.1**) and were significant for facilitating the survival of HE06 *SS2* under neutral and acidified growth condition *in vitro*.

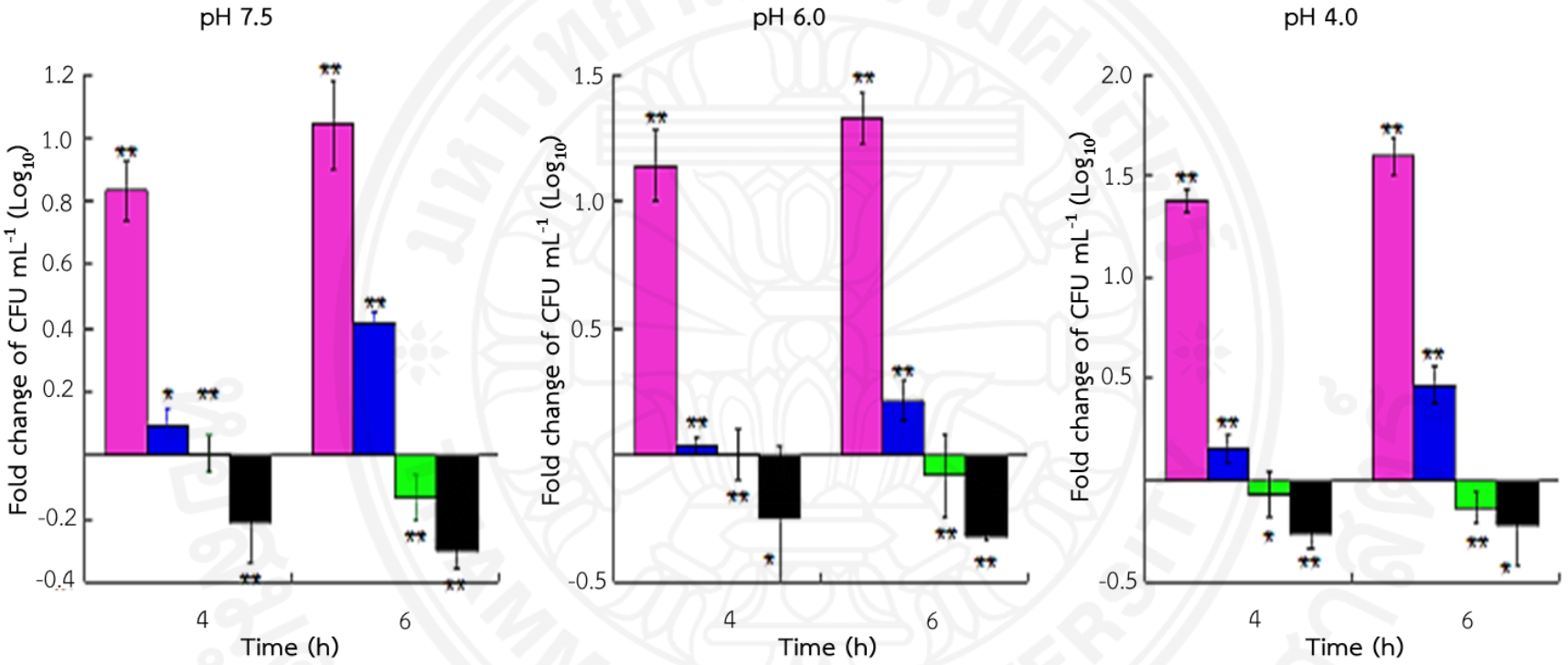

Figure 5.43 Growth of HE06 *SS2* in DMEM. The pink columns represent DMEM supplemented with 10 mM L-arginine. The blue columns represent DMEM without L-arginine. The green columns represent DMEM supplemented with 20 mM L-canavanine. The black columns represent DMEM supplemented with 0.5 mM L-NIO. The results are mean values obtained from three independent experiments. *p*-value was calculated by comparing to the fold change of CFU/ml obtained from inoculum sample. * indicates *p*-value < 0.05, ** indicates *p*-value < 0.01. Left panel represents DMEM pH 7.5. Middle panel represents DMEM pH 6.0. Right panel represents DMEM pH 4.0

CHAPTER 6
DISCUSSION

Streptococcus suis is the cause of a severe zoonotic infection in humans, especially in the Northern region of Thailand, characterized by septicemia, meningitis and endocarditis. In the past 10 years, the incidence of human *S. suis* infections in Thailand has substantially increased. Moreover, many retrospective descriptive studies have indicated the involvement of pigs or their products as the most probable cause of infection in epidemic and sporadic human cases in this country.[20,71] Previous reports have demonstrated that only a few cases of STSLS, similar to those of the *S. suis* ST7 Chinese outbreak strain, had been reported in Thailand, providing a hint in pathogenicity differences.[71] However, the information regarding the genetic link of *S. suis* recovered from human patients and pig isolates has remained unknown. The experiments described in this thesis provided data on biochemical profile and *S. suis* serotype that are useful for conventional identification in diagnostic laboratory in Thailand. Moreover, the genetic characteristics of 245 *S. suis* isolates recovered from either human patients or pigs during the 2007 human outbreak in Thailand in both endemic (Northern) and other non-endemic regions of Thailand were revealed. The genetic relationships among *S. suis* isolates from both pigs and humans in Thailand and their virulence gene profiles also had been addressed. Moreover, recombinant *SS2* ArcA expression in *E. coli* clone was constructed and its physical properties and biological function was elucidated. Mutagenesis of *S. suis arcA* was pursued. Inactivation of ArcA enzyme in *S. suis* using specific inhibitor resulted in reduction of *S. suis* survival in acidic environment *in vitro*. This warrant the contribution of *S. suis* ArcA in their intracellular survival in eukaryotic host cells.

6.1 *S. suis* identification and serotypes

Using the conventional biochemical system for identification of presumptive *S. suis* and further confirmed the specie by using *gdh* PCR indicated that the majority of *S. suis* Thai isolates (87%; 212 of 245 isolates) collected from humans and pigs regardless of their serotypes were positive for both trehalose and raffinose (71%) or trehalose alone (15%). Moreover, all of these conventional identified *S. suis* and probable *S. suis* were *gdh* PCR-positive, therefore fermentation of trehalose and raffinose could be a reliable biochemical markers for *S. suis* identification in Thailand (**Table 5.1**). Although the minor *S. suis* population collected from pig samples which conventionally identified into the doubtful *S. suis* isolates (belonging to serotypes 16 to 34, AA and NT), in this study were unable to ferment both trehalose and raffinose, their specie could confirm by the presence of *gdh* by PCR. These different biochemical characteristics were also previously described.[41] It was reported that approximately 4–7% and 12–19% of *S. suis* isolates belonging to serotype 9 to 22 were negative for both trehalose and raffinose. On the other hands, among serotypes 1 to 8 less than 6% and 10% of isolates were negative for those both sugar fermentations. Moreover, these doubtful *S. suis* isolates were positive only for the presence of *S. suis*-specific *gdh* sequence but not *S. suis*-specific 16S *rDNA* sequence. These data suggesting that it was essential to perform *gdh* PCR for *S. suis* speciation in the pig isolates giving doubtful result in identification by the conventional method. In addition, only 71% of *S. suis* in this study (175 of 245 isolates) were 16S *rDNA* positive compared to *gdh* PCR. Therefore, the data in this study further confirmed that PCR targeting *S. suis*-specific *gdh* sequence[43] was providing a higher sensitivity than 16S *rDNA* for Thai *S. suis* identification.

The capsular types of more than half the *S. suis* isolates collected from the pig origins in this study (131 of 245 isolates) were unknown due to their autoagglutinating appearance even prior to adding the anti-capsular antiserum or unable to co-agglutinate with any tested anti-capsular antiserum. However, *cps2*-PCR amplification demonstrated that these isolates were not belonged to serotype 2. It was possible that these isolates were unencapsulated or carrying a not yet identified

S. suis capsular antigens. The previous study showed that almost 5% of *S. suis* isolates recovered from diseased animals and majority of the isolates recovered from the tonsils and nasal cavity of healthy animals in Korea, Canada and France were unencapsulated.[35,157–160] Among the 21 identified serotypes in this study, the serotype 2 is the most prevalence (14.69%) followed by serotype 22 (20 isolates, 8.16%). Although, serotype 22 *S. suis* has been reported to involve with septicemia, meningitis, and sudden death in pigs, they had not yet been reported for causing human zoonotic diseases in Thailand, China and Vietnam. In addition, approximately three to seven isolates belonging to serotypes 3, 5, 9, 16, 29, 30 and 34 were reported among pig samples in this study. The serotypes 16 and 9 *S. suis* were prevalence among healthy pigs in Canada and Korea.[159,161] However, both serotypes were found in both diseased and healthy pigs in this study. Moreover, serotypes 17, 18, 19 and 21 which were generally reported from healthy pigs elsewhere were rarely found in this study.[40,161] These data indicated apart from the predominant of serotype 22, the *S. suis* isolates from either diseased or healthy pigs in Thailand were belonged to a variety of serotypes.

Interestingly, one isolate of each serotype 1 and 14 from diseased pig in Nakhon Pathom and Nakhon Si Thammarat, were found. These serotypes have been associated with the previously reported patients in Thailand as well as Southern Vietnam and North America. Therefore, it should be emphasized that all *S. suis* serotypes associated with previous reported human zoonosis were presence among illed and healthy pigs in Thailand, which indicates the potential for the anthropozoonotic transmission.

6.2 Virulence genes and genetic diversity of *SS2* Thai isolates

S. suis often mediate severe zoonotic infection in Thai patients, especially in Northern part of country as a consequence of septicemia, meningitis and endocarditis. However, only a few cases of STSLS similar to that of the Chinese *SS2* outbreak had been reported.[71] In the past 10 years, the incidence of *S. suis* infection in Thailand has substantially increased. Moreover, many retrospective

descriptive studies in Thailand indicated the involvement of pigs or their products in epidemic and sporadic patients.[20,71] However, the information on genetic linkage of *S. suis* among patients and pigs isolates remained unknown. In this study, genetic characteristics of 245 *S. suis* isolates recovered from either human patients or pigs during the 2007 human outbreak in Thailand in both endemic (Northern) and other non-endemic regions of Thailand were studied. A total of 36 isolates belonged to serotype 2 as identified by PCR and serotyping (**Table 5.2**). All human isolates in this study were serotype 2, while only 2 out of the 6 isolates collected from diseased pigs in a non-endemic zoonotic area (Nakhon Pathom) and 7 of the 194 isolates recovered from healthy pigs in an endemic area (Phayao) belonged to this serotype (**Table 5.3**). None of the *S. suis* isolates recovered from diseased pigs in the Phayao (endemic) and Nakhon Si Thammarat (non-endemic) regions were serotype 2. The lack of *SS2* isolates in diseased pigs in Phayao and the fact that only 2 isolates of this serotype were isolated from Nakhon Pathom was interesting. As of 2007, almost 5 million pigs in Thailand (representing 60% of the swine population) were being reared in large hygienic farms of intensive swine production industries in the *S. suis*-zoonotic-free Central Plain area, particularly in the Nakhon Pathom Province. Meanwhile, only 1.7 million pigs were domestic and reared in small farms in the Northern region.[162] However, from 2005 to 2006, only one *S. suis* case was reported in a diseased pig specimen from the Central Plain Provinces submitted to the microbiology laboratory of the National Institute of Animal Health, Thailand (NIAH).[163] As such, it is likely that *S. suis* infections in pigs in the Central Plain Provinces, as in other areas of the country, are virtually unknown and, most probably, underestimated.

The *epf*, *mrp* and *sly* genes have been shown to be significant virulence markers for *S. suis* that allow to differentiate virulent (presence) from less-virulent (absence) strains in Europe and Asia.[16] The presence of other additional genes, such as *arcA*, *bay046*, and *hyl*, has been also reported to be associated with *S. suis* virulence.[9–11] The results of the present study revealed that the majority of *SS2* isolates regardless of their origin (human or pig), epidemiological status (epidemic or sporadic) and disease category (diseased or healthy pigs), carried all virulence-

associated genes tested (VAGP1). The *SS2* isolates with the *epf*$^{+}$/*mrp*$^{+}$/*sly*$^{+}$ genotype had also frequently been described from cases of meningitis in patients from Thailand, Vietnam and China, as well as from the majority of diseased pigs during the largest zoonotic outbreak in China, in 2005.[17,18,70] Moreover, isolates that produce the EF, MRP and SLY were frequently associated with *SS2* from diseased pigs in Europe.[13] A total of five of the seven *SS2* isolates from healthy pigs possessed also the *epf*$^{+}$/*mrp*$^{+}$/*sly*$^{+}$ genotype indicating that these isolates carried the potential virulence to infect humans. On the other hand, two *epf*$^{-}$/*mrp*$^{-}$ (VAGP2) isolates, from a sporadic non-meningitis patient and a diseased pig, respectively, had similar genotypes to those of *SS2* isolates recovered from non-meningitis patients in Thailand and from diseased pigs in North America, as previously reported.[17,164,165] Finally, a healthy serotype 2 pig isolate in this study was identified as *mrp*$^{-}$ (VAGP4), similar to *SS2* strains isolated from meningitis patients in Vietnam.[18] Taken together, our results support previous reports indicating that the EF and MRP may be significant virulence markers while not being essential for pathogenesis in pigs and humans.[12] A previous report suggested that RAPD clusters obtained with the OPB7, OPB10 and OPB17 primers were usually related to a given EF, MRP and SLY phenotype, rather than to the geographic or host origins of the strains.[13] Moreover, RAPD clusters indicated that *SS2* isolates from North America and Europe might have originated from a common ancestor.[13] The identical RAPD pattern and its specific virulence gene profile were also found in most clinical *SS2* isolates from Brazil indicating the successful spread of a specific clone to South America.[14] Similar results were obtained in the present study. The four RAPD obtained patterns (RAPD-A to RAPD-D) were also related to their own specific virulence gene profile (VAGP). RAPD-A patterns obtained with the OPB7 and OPB17 primers were solely located on the Cluster 1 of the dendrogram and not related to other patterns, whereas, using the OPB10 primer, RAPD-A, RAPD-B and RAPD-C were related and grouped together in Cluster 1, suggesting differences in the discriminatory power of these OPB primers. Most of *SS2* isolates collected from human patients (epidemic and sporadic) and pigs (diseased and healthy) in different regions of Thailand had a RAPD-A/VAGP1 pattern,

suggesting a close genetic relationship among human patients and diseased and healthy pig isolates. This phenomenon was further emphasized by the MLST results. The majority of the *SS2* RAPD-A/VAGP1 isolates in this study were classified as ST1. This ST is also present in isolates from Vietnam, Hong Kong and Europe as well as a few isolates from North America.[18,21] Moreover, *SS2* ST1 strains have been previously reported to be a frequent cause of meningitis in human patients in Thailand.[17] In addition, five of the seven *SS2* isolates from healthy pigs were found to be ST1, which was similar to that of the isolates from slaughterhouse pigs in Southern Vietnam.[166] These results further emphasize the successful establishment of the *SS2* ST1 clone in Asia.[16] Two RAPD-B/VAGP2 *SS2* isolates from a diseased pig and a non-meningitis human patient were identified to be ST104, a result that is similar to a recent report identifying this ST as a new and unique sequence type found in meningitis patients in Thailand.[17] Although, these isolates were *epf*¯*/mrp*¯ (VAGP2), similar to those of the *SS2* isolates from diseased pigs in North America, their ST was different.[21,165] The *epf*¯*/mrp*¯ North American *SS2* isolates were ST25, which may explain the low incidence of human zoonotic infections in that continent when compared to Thailand. This information indicates that there is a selective pressure on the *S. suis* ST1 and ST104 clones in pigs to be zoonotically transmitted to humans in Thailand. In this study, two unique STs were found in healthy serotype 2 pig isolates: one with an *epf*¯*/mrp*¯*/sly*¯ genotype (VAGP3), belonging to the RAPD-C pattern, was identified as ST233, and the other as VAGP4 (*mrp*¯)/RAPD-D was identified as a newly discovered ST, ST336. The latter ST was assigned to a separate branch of the dendrogram. To date, *SS2* clones associated with zoonotic disease in the different regions of the world appear to be unique and diverse based on MLST results.[16,17] Although, an ST233 and a new ST336 isolate were only collected from healthy pigs, their RAPD patterns revealed similarities to isolates with RAPD-B pattern recovered from human and diseased pig. Therefore, their zoonotic potential can not be excluded. In conclusion, the results obtained in this study indicate that virulence gene profiles can be correlated with both the RAPD patterns and STs for different *SS2* isolates in Thailand. Our findings, the first pertaining to isolates from Thailand,

partially elucidate the genetic relationships and confirm the zoonotic transmission of *S. suis* isolates from pigs to human for certain STs such as ST1 and ST104.

6.3 Virulence genes and genetic diversity of non-*SS2* Thai isolates

S. suis had been known as an important pathogen in newborn piglets and fattening pigs as well as adult pigs that have been raised in poor hygienic condition. The organism causes a wide variety of diseases in young pisg including meningitis, endocarditis, arthritis and pneumonia.[1,2,167] Adult pigs healthy carried *S. suis* in their upper respiratory tract, particularly the palatine tonsils and nasal cavities. The genital and alimentary tracts of pigs were also the sites for *S. suis* colonization. The highly pathogenic *SS2* colonized palatine tonsils of both clinically ill and healthy pigs. Importantly, the 80% of the adult pigs healthy carried of this organism and were the potential source of infection to other animals in the herd and humans.[168] To date, in Thailand, very little is known about the serotypes of *S. suis* that could cause the infection in diseased pigs. Moreover the virulence gene profile and genetic characteristic of the pig isolates were also unknown. This study revealed that non-*SS2* isolates carried three VAGPs (1, 2 and 4) that presented in *SS2* isolates and other 10 additional VAGPs, *i. e*., VAGP5 to VAGP14 (**Table 5.4**). Half of non-*SS2* isolates (44.02%, 92 of 209 isolates) collected exclusively from healthy pigs carried only *bay046* and *arcA* designated as VAGP11 (**Table 5.4**). This genotype was *epf*$^{-}$/*mrp*$^{-}$/*sly*$^{-}$ similar to most of *S. suis* isolates from diseased and healthy pigs in Asia, Europe and North America.[169] It is believed that this VAGP was less virulence than *epf*$^{+}$/*mrp*$^{+}$/*sly*$^{+}$genotype for both human and pig infection.[13] Interestingly, majority of diseased pig isolates in this study carried the previously recognized low pathogenic traits (*epf*$^{-}$/ *mrp*$^{-}$/*sly*$^{-}$) *i. e.,* 6 out of 22 isolates carried VAGP12 (positive for *bay046* only) and 14 of 22 diseased pig isolates carried VAGP14 (negative for all six tested putative virulence genes and markers). Furthermore, all isolates belonging to serotypes 7 and 9 (nine isolates) in this study, in Europe and North America were negative for *epf* America[4,136], whereas the majority of slaughterhouse pigs isolates in

China carried *epf* gene. These findings further supported the previous information that the presence of *epf*, *mrp* and *sly* was varied depending on the geographical regions and could be considered only as a virulence markers rather than the virulence factors as the mutagenesis in these three genes unaffected pig infection.[170] Therefore, the presence of those VAGPs as mentioned above indicating that these three markers was not useful for prediction of the virulence of the non-*SS2* isolates in Thailand. Moreover, it should be emphasized that *epf*, *mrp* and *sly* were absence from over 90% of the non-*SS2* diseased pig isolates (20 of 22 isolates), which indicates that additional virulence factors apart from the six tested genes are responsible for virulence in pigs. In addition, VAGP1 (*SS1*; 1 and *SS14*; 1), VAGP1 (NT; 1), and VAGP2 (*SS3*; 1, *SS7*; 2, *SS28*; 1, *SS34*; 1 and AA; 2) which considered as the genetic traits possessed the high anthropozoonotic transmission potential, were carried by two diseased, one healthy, and seven healthy pig isolates, respectively. These data suggesting that the minority of non-*SS2* isolates may have a potential to infect human.[171]

The genetic diversity of non-*SS2* isolates were studied by using RAPD PCR with primers OPB7, OPB10 and OPB17. Initially, dendogram based on RAPD patterns obtained from all 209 non-*SS2* isolates were constructed. The dendogram trees obtained from the individual three RAPD primers revealed numerously individual branches indicating that all of the non-*SS2* isolates carried by pigs in Thailand in this study is extremely diverged (**Figure 5.4**). Thereafter, dendogram based on RAPD patterns obtained from 16 non-*SS2* isolates carrying VAGP1 to VAGP4 were constructed and the results showed that majority of these isolates were substantially related. Moreover, they were also related to the *SS2* isolates that carrying the same VAGPs, although, the relationship was not closely (**Figure 5.5**). These results further emphasized that similar to the previous finding for the *SS2* isolates, the RAPD patterns of the non-*SS2* isolates also link to their VAGPs.[13,14] Furthermore, these relationships were not associated to the serotype of the non-*SS2* isolates. Taken together with the mentioned above, apart from sharing the VAGPs, these 16 non-*SS2* isolates also carried related genetic background to the high zoonotic potential *SS2* isolates indicating that non-*SS2* isolates carrying a potential to infect human[171] were

actually presented in pigs in Thailand. In addition, the data above emphasized on the significant of the screening for the presence of putative virulence gene profiles and genetic diversity of the non-*SS2* isolates in pigs rather than the serotype for the prevention and control of non-*SS2* zoonotic infection in Thailand in future.

6.4 Expression and characterization of arginine deiminase

Arginine deiminase (ArcA or ADI) of *SS2* is one of many putative virulence factors. It might involve in intracellular survival of *S. suis* under acidic conditions similar to their orthologues in other pathogens. Moreover, ArcA is indeed expressed *in vivo* during *S. suis* infection process (Srimanote *et al*, unpulished data and Jing *et al* [22]). However, the biological properties and function of *SS2* ArcA have not yet been characterized. Therefore, the study in this section aimed to characterize physical and enzyme kinetic properties of ArcA in *SS2*. The *arcA* gene of *SS2* was successfully cloned into pET protein expression system. The *E. coli* clone carrying pET23a+::*arcA* was successfully induced to express an enzymatically active soluble r*SS2* ArcA using T7 RNA polymerase/promoter two plasmids system. Due to the standard imidazole elution inhibited ArcA activity, EDTA elution buffer was used for protein purification. Although, eluting nickel IMAC column with EDTA buffer is known to strip the nickel ion from the column and eluted with recombinant protein, these nickel ions had no impact on ArcA activity. As a consequence of intact r*SS2* ArcA activity after treatment the crude r*SS2* ArcA prepared from lysate of heat induced *E. coli* clone carrying pET23a+::*arcA* with 10 to 100 mM of nickel sulfate solution (**Appendix J**). This purification system was also previously carried out in *P. putida* ArcA purification.[153] As shown in **Section 5.4.2**, the yield of r*SS2* ArcA protein purification was quite low (34.19%), however, this single-step affinity-based purification procedure provided a very high purity (266.67-fold purification) as indicated by their specific enzymatic activity. In addition, there is no difference on r*SS2* ArcA activity followed the dialysis to eliminate EDTA indicating that the presence or absence of EDTA in r*SS2* ArcA solution had no effect on r*SS2* ArcA activity (data not shown). The purified r*SS2* ArcA was verified by LC-MS/MS analysis and the results confirmed that both 47 and 25 kDa

of protein band as shown in SDS-PAGE of the purified recombinant protein obtained in this study was ArcA of *SS2*.

Multiple alignment of deduced amino acid sequence of *SS2* ArcA demonstrated a high sequence similarity to *S. pyogenes* (91%). Although *SS2* ArcA shared a weak similarity (36%) to the well-characterized *Mycoplasma* ArcA (36%), it possessed the four critical highly-conserved motifs and the catalytic triad characteristic of guanidino group-modifying enzyme superfamily. Cys399 within catalytic site is responsible for the nucleophilic attack which consists of the Cys-His-Glu triad.[148] A drastic loss of *SS2* ArcA enzymatic activity was observed under a non-reducing condition (data not shown). In addition, the difference in optimum temperature and pH of ArcA activity was observed between the assay reaction with and without DTT. This study confirmed the previous evidence reported by Cugini *et al*.[172] that ArcA activity was rapidly lost in non-reducing environment and the addition of DTT greatly increased enzymatic activity. These results indicated that the *SS2* ArcA was highly sensitive to oxidizing condition and Cys399 residue could play an essential role in the *SS2* ArcA catalysis. Although the ArcA of different organisms possesses nearly the same numbers of amino acid (406–420 residues) as shown in **Table 5.6**, the difference of ArcA protein oligomerization could lead to the varieties of apparent molecular weight (MW) of ArcA proteins in their native forms. The three-dimentional structure of *P. aeruginosa* ArcA demonstrated a homotetramer with MW of 184 kDa.[33,173] Although the amino acid sequence alignment between *P. aeruginosa* ArcA and *P. putida* ArcA demonstrated a high similarity (84.21%), they were distinct in protein oligomerization. Unlike *P. aeruginosa* ArcA, the ArcA of *P. putida* could fold into a homodimer with MW of 130 kDa.[153] Upon the high MW of *L. buchneri* NCDO001 ArcA (MW = 199 kDa), it is likely that the protein could exhibit a homotetrameric form.[152] It has been shown that *S. pyogenes* exhibits dimeric ArcA with MW of 100 kDa[121] and *M. arginini* ArcA structurally folds into a homodimer with MW of 90 kDa.[174] The homotrimer of ArcA (MW=138 kDa) could be also found in *L. lactis* ssp *lactis* ATCC 7962.[151] Taken together, the data suggested that the native folding of ArcA was diverse in many organisms. In this study, the SDS-PAGE analysis revealed the purified r*SS2* ArcA with MW~47 kDa (**Figure 5.16**). In addition,

the native PAGE analysis of cell lysate and purified r*SS2* ArcA demonstrated a major intense band of 47-kDa *SS*2 ArcA (data not shown). These data suggested that of *SS2* ArcA could functionally active as a monomeric form. However, whether the protein folding of *SS2* ArcA could introduce the oligomerization of active *SS*2 ArcA remains elucidated.

The optimum temperature of ArcA in *P. aeruginosa*, *B. cereus*, and *E. coli* is 25 ºC while the high optimal temperature at 50 and 60 ºC was observed in *L. buchneri* NCDO110, *M. arginini*, *P. putida* and *L. lactis* ssp lactis ATCC 7962. Taken together, the data indicated that the ArcA could function in a wide range of host environment. Although the amino acid sequences alignment of ArcA revealed a high similarity between *S. pyogenes* ArcA and *SS2* ArcA (89.98% identity, **Table 5.6**), they exhibited the different optimal temperature, 37 and 42 ºC for *S. pyogenes* and *S. suis* serotype 2, respectively. In addition, a previous study from Winterhoff *et al*.[9] showed that the up-regulation of *SS2* ArcA was significantly affected by temperature stress.[9] Interestingly, these results showed that the ArcA of *S. suis* could be enzymatically active in a wide range temperature, representing the physiological temperature for bacterial colonization at the upper respiratory tract (30 ºC), bacterial invasion of deep tissue (37 ºC) and fever (42 ºC). Hence, it could be postulated that the ArcA of *SS2* might have a significant role in the infected host during pyrogenic stage. For the pH dependency, r*SS2* ArcA could functionally active in a wide ranges pH between 4.2 and 7.5 suggesting the ArcA of *S. suis* prefers the acidic environment for its function. During the phagocytosis mechanism, the acidic pH range in early phagosome, late phagosome, and phagolysosome is pH 6.0–6.5, pH 5.0–6.0 and pH 4.5–5.0, respectively.[175] Whereas the pH condition of intracellular fluid, extracellular space, and human blood is 7.0, 7.2 and 7.35–7.4, respectively. Based on the results of pH dependent activity of r*SS2* ArcA *in vitro*, it could be postulated that the ArcA of *S. suis* could functionally active throughout the phagocytosis mechanism. Moreover, these data supported the previous information from Gruening *et al*. that the ArcA could facilitate *S. suis* survival in acidified environments.[34] The results of thermostability shown in **Figure 5.20** indicated the stability of r*SS2* ArcA at 4 and 25 ºC for 3 h. The r*SS2* ArcA activity remained approx. 80% activity for 2 h at 30 ºC. At

37 ºC pre-incubation, the r*SS2* ArcA activity reduced to 50% within an hour and completely diminished after 2 h of pre-incubation. At 42 ºC pre-incubation, the activity of r*SS2* ArcA remained 50% within 30 min and significantly lost after 2 h of pre-incubation. Although the r*SS2* ArcA could functionally active at 42 ºC, the r*SS2* ArcA was temperature sensitive. It is likely that the thermostability properties of the microbial enzyme could be introduced by the physiological temperature of microbial sources. As shown in previous evidences, a common bacteria used for diary production, *L. lactis* could retain enzymatic activity more than 80% at 40 ºC for 2 h[151] and thermophilic fungi, *A. fumigatus* could resist to thermal denaturation at 50 ºC.[155] Compared to the thermostable ArcA, the amino acid sequence of the *SS2* ArcA showed less similarity with *A. fumigatus* (7.14%). The sequence alignment shown in **Figure 5.15** clearly revealed the differences of highly conserved regions and flanking amino acids between *SS2* ArcA and *A. fumigatus* ArcA. In addition, the number of amino acid residues of *A. fumigatus* ArcA (308 residues) is less than that of *SS2* ArcA. In addition, *A. fumigatus* ArcA contains seven Cys residues that are significantly higher than that of *SS2* ArcA containing two Cys residues. The data suggested that the difference of amino acid content could lead to the structural integrity and conformational stability of the enzyme. However, the determinants of intrinsic thermostability and thermal catalysis properties for *SS2* ArcA remain further investigated. Although the enzymatic activity of *SS2* ArcA could be reduced after pre-incubation at 37 ºC, it was more stable at acidic condition than basic condition. This evidence supported that the r*SS2* ArcA was capable to play a key role in acidic environment of phagocytic processes.

The kinetic values of different ArcA were shown in **Table 5.14**, indicating that a substrate binding affinity of ArcA was highly variable among different species. Although the amino acid sequence alignment revealed a high sequence similarity at conserved regions and catalytic site of r*SS2* ArcA and that of *S. pyogenes* ArcA, their K_m values were significantly different, suggesting that the structural integrity of non-conserved regions might contribute to the substrate binding affinity. Both ArcA and nitric-oxide synthase (NOS) could utilize arginine as substrate and the requirement for arginine could be varied considerably in different organism. Many evidences

supported the effect of the ADI on diverse roles of nitric oxide synthesis in various biological systems including both innate and adaptive immune responses.[155,173,176] In mammalian cells, it has been shown that the treatment of purified recombinant ADI could suppress NO production[112,173] and the deprivation of L-arginine level through ADI could interfere nitric oxide (NO) synthesis in phagocytic cells.[177] In *S. suis*-infected cells, the effect of *SS2* ArcA on the regulation mechanism of NO synthesis remains discovered.

The chemical mechanism for L-canavanine inactivation of ArcA has been well characterized in *P. aeruginosa*, *E. coli*, *Burkholderia mallei*, *B. cereus* and *Giardia intestinalis*.[154] The ArcA inhibition by L-canavanine in different species was summarized in **Table 5.14**. In dept kinetic study and mass spectrometry-based analysis clearly demonstrated that the L-canavanine irreversibly inactivates the ADI of *B. cereus*, whereas the L-canavanine inactivation of *P. aeruginosa*, *E. coli*, *B. mallei* and *G. intestinalis* ADIs is reversible and time controlled reaction.[154] One possible explanation could be the divergence of structural conformation; the superposition of 3-D structures revealed a larger disorder of two loop regions around the active site of *B. cereus* ADI, compared to that of *P. aeruginosa* ADI.[154] It is likely that the loop regions of *B. cereus* ADI are more flexible than that of *P. aeruginosa* ADI, contributing to the diverse ADI inactivation by L-canavanine. In comparison with *B. cereus* ADI, the residues involved in the substrate and L-canavanine binding present in the active site of *SS2* ArcA are highly conserved in the active site of *B. cereus* ADI. However, our result showed that the *SS2* ArcA exhibits a considerably lower binding affinity to L-canavanine. For another inhibitor, L-NIO was the most potent inhibitor for NO synthase in phagocytic cells such as neutrophils and macrophages.[156] In addition, NO synthase that has L-arginine as substrate similar to ArcA, thus this inhibitor was also used for testing the inhibition assay of ArcA. The results were quite similar to use the L-canavanine inhibitor due to the lack of 100% enzyme inhibition as a reference point. Both of these L-arginine analogues were not the good inhibitor for r*SS2* ArcA, thus more structural information and kinetic data are required to clarify the distinct binding behavior in order to define the reaction of L-canavanine or L-NIO inaction of *SS2* ArcA.

6.5 The contribution of *SS2* ArcA in the survival of *S. suis* under acidic condition

The expression of crude native *SS2* ArcA from HE06 *SS2* was successfully induced using low glucose DMEM without L-arginine tissue culture medium. The supplement of 10 mM L-arginine in DMEM resulted in approximately two folds induction of *SS2* ArcA expression at 4 and 6 h as determined by enzyme activity assay but different in *SS2* ArcA quantity were not observed in Western blot analysis. The ArcA from many lactic acid bacteria were also previously shown to be induced in L-arginine containing medium.[34]

Testing of physical and kinetic properties of crude *SS2* ArcA revealed that its stability was extended toward the higher temperature than that of the purified r*SS2* ArcA but their optimum temperature and pH for enzymatic activity were demonstrated to be similar (42 ºC and pH 7.2) (**Sections 5.5.2** and **5.4.7.1**). It was possible that in the presence of other *SS2* cellular protein species, *SS2* ArcA could better preserve their active conformation while temperature was increasing. These results were further supported by the finding that *SS2* ArcA was enzymatically active in a wider range temperature in the presence of their substrate. In thermostability assessment of r*SS2* ArcA and crude native *SS2* ArcA (**Figures 5.20** and **5.27**) revealed that both enzymes were equally well stable when kept at 4, 25 and 30 ºC for 3 h, however, the crude native *SS2* ArcA exhibited their full activity while the purified r*SS2* ArcA lost 50% their activity after 1 h pre-incubation at 37 ºC and their activity were completely diminished after 2 h of pre-incubation. At 42 ºC pre-incubation, both enzymes significantly lost their activity after 2 h of pre-incubation. These results also suggested the role of other *SS2* cellular proteins in maintaining the active conformation or protect the active site of active ArcA at 37 ºC and above temperature in an absence of L-arginine substrate. Moreover, these data also suggesting the possibility that *SS2* ArcA activity might significant for *S. suis* pathogenesis steps *in vivo* such as normal mammal body temperature (37 ºC), pyrogenic stage and inflammatory site (above 37 ºC) and septic shock (below normal body temperature). The enzymatic activities of both crude native *SS2* ArcA and r*SS2*

ArcA were stable when kept in acidic to neutral condition but they were alkali pH sensitive as their activities were almost abolish at pH 8.0 (**Figures 5.19** and **5.26**). Moreover, both crude native *SS2* ArcA and r*SS2* ArcA had the same optimal pH for their enzymatic activity at neutral or slightly acidic (pH between 4.2 and 7.5) suggesting the preference for function in acidic environment.

In addition, while comparing the enzymatic activity results between temperature dependency and temperature stability of r*SS2* ArcA and crude native *SS2* ArcA, it revealed a significant effect of arginine substrate binding to *SS2* ArcA leading to catalyze the enzymatic reaction and preserve the activity of these enzymes. As shown in **Figures 5.21** and **5.28**, in an absence of L-arginine, the crude native *SS2* ArcA was fully stable only under pH 4.2–5.0 at 30 ºC for 3 h but their activity was disappear when pre-incubate at pH 7.2 for 3 h whilst their maximum activity was exhibit at pH 7.2 in the presence of L-arginine substrate.

More interestingly, in an absence of L-arginine the enzymatic activity of crude native *SS2* and r*SS2* ArcA reduced after pre-incubation at 37 ºC under the neutral to alkali pH, however the better stability were demonstrated at 37 ºC under acidic pH (4.2–6.0). This evidence supported that the *SS2* ArcA harbor the characteristic to possibly play a role in acidic environment with the particular reference to the survival of *S. suis* in the phagosome following the engulfment by phagocytes. Based on all of results in physical properties of r*SS2* ArcA and crude native ArcA in *S. suis*.

By using non-linear regression analysis, the K_m and V_{max} values of crude native *SS2* ArcA were conducted similar to the r*SS2* ArcA, K_m of crude native *SS2* ArcA (0.157 mM) at 42 ºC were lower than r*SS2* ArcA (0.176 mM) indicating that a substrate binding affinity of crude native ArcA was lower than the purified r*SS2* ArcA. However, compared to its orthologous ArcA proteins, crude native *SS2* ArcA still possessed a relatively high affinity for arginine substrate similar that of the other previously characterized ArcA (**Table 5.14**). This was not surprised as a result of the purity of purified r*SS2* ArcA rendered their active site had a high opportunity to bind with substrate L-arginine substrate. Moreover, deduced amino acid sequence analysis of alignments revealed that the Cys399 residue laid in ArcA catalytic site was only 16

amino acid residues far away from the C-terminal hexahistidine tag, therefore, it was possible that this positive charge amino acids stretch may facilitate the better substrate binding of r*SS2* ArcA. In order to assess the involvement of hexahistidine tag in L-arginine substrate binding of r*SS2* ArcA in the future, it will require the construction and the expression of r*SS2* ArcA without C-terminal hexahistidine tag.

The inhibition assay of crude native *SS2* ArcA by using L-canavanine and L-NIO revealed that like r*SS2* ArcA inhibition assay, these inhibitors could only partially inhibit crude native *SS2* ArcA. Demonstrating that both L-arginine analogues were not an ideal inhibitor for both crude native *SS2* ArcA and r*SS2* ArcA because these enzymes had only the weak binding affinities to the L-canavanine and L-NIO inhibitors compare to their actual L-arginine substrate. For complete in inhibition of *SS2* ArcA activity, the new inhibitor should have the larger molecule than L-arginine in order to facilitate the better competitive capability with the actual substrate yielding the higher binding affinity that the existing L-canavanine and L-NIO inhibitors.

Confirmation that *SS2* ArcA was required for *S. suis* survival under acidic environment was initially achieved by construction of a defined *arcA* negative mutant. Unfortunately, the survival of wild type *SS2* compared to its isogenic *arcA* negative mutant derivative in the acidic condition was not performed in this study because of the inability to construct *arcA* negative mutant. In this work, attempts to construct *arcA* negative mutant using pVA891 and pSET4S vectors were similarly unsuccessful. Alternatively, although L-canavanine and L-NIO provided only partial inhibition for crude native *SS2* ArcA, it possessed the specific binding to *SS2* ArcA in *SS2*. Therefore, these inhibitors were used to inactivate the *SS2* ArcA in order to demonstrate its role in *S. suis* survival under acidic environment.

Growing of *S. suis* in the DMEM supplemented with 10 mM L-arginine, *S. suis* expressed ArcA higher than growing in DMEM without L-arginine under pH 4.0, 6.0 and 7.5 representing the pH in phagosome during the phagosomal maturation step resulted in 2.97 ($p < 0.01$), 1.71 ($p < 0.01$) and 2.62 ($p < 0.01$)-fold increasing in number of *S. suis* survival at 6 h incubation, respectively. Moreover, inactivation of *SS2* ArcA using L-canavanine or L-NIO resulted in approximately 0.72 ($p < 0.01$), 0.84 ($p < 0.01$) and 0.75 ($p < 0.01$) or 0.63 ($p < 0.01$), 0.48 ($p < 0.01$) and 0.63 ($p < 0.01$)-

fold reduction in number of *S. suis* survival at 6 h incubation compared to the inoculum number, respectively. In addition, the significant reduction in number of *S. suis* survival under all tested pH and incubation periods in the DMEM supplemented with L-canavanine or L-NIO inhibitor relative to those obtained from DMEM supplemented with 10 mM L-arginine. It should be emphasized, however, that in the presence of inhibitors and absence of L-arginine the reduction in number of *S. suis* survival under all tested pH and incubation period was statistically significant ($p < 0.01$) but did not exceeded 100-fold reduction. These results were certainly attributed to the consequences of only half of ArcA activity was inhibited by the two inhibitors and the low background expression of ArcA in *S. suis* growing in the DMEM without L-arginine (**Figure 5.43**). These results further supported by the previous study, which elucidated that only the NH_3 produced by ArcA alone rather than the whole ADS were able to provide the substantially longer survival of oral streptococci in acidic environments.[24,25] These oral bacteria require NH_3 product of ADS to neutralize the local acidity derived from sugar metabolism following the oral biofilms formation.[24–26] Furthermore, both NH_3 and ATP productions from ADS were essential for streptococci adaptation in acidic environment in both extra- and intracellular life style.[178] In conclusion, the work in this thesis has contributed to our knowledge of *SS2* ArcA characteristic and partly elucidated its preliminary role in facilitating *S. suis* survival under acidic environment *in vitro*, however, there will be more experimentation to fulfill before the role of *SS2* ArcA in *S. suis* pathogenesis will be fully understood.

CHAPTER 7
CONCLUSION

This study has pursued to provide an understanding into the role of arginine deiminase (ArcA) in pathogenesis mechanism of *SS2*. The prevalence of *arcA* gene and other virulence markers as well as the genetic diversity of *S. suis* Thai isolates were investigated. The findings in this thesis can be concluded as the following:

1. In this study, a total of 245 *S. suis* isolates was collected from both human cases (epidemic and sporadic) and pigs (diseased and healthy) in Thailand.

2. Identification of *S. suis* strains, in particular the 36 *SS2* isolates regardless of the source of isolation by conventional biochemical tests completely conformed to PCR targeting *gdh* gene (**Table 5.1**).

3. Serotype 22 was the most prevalence non-*SS2* serotype in this study. The conventional biochemical tests remained effective for identification of the majority of non-*SS2* isolates in this study (**Table 5.1** and **5.2**).

4. *arcA* has a high prevalence (168 out of 245 isolates, 68.57%) in *S. suis* Thai isolates compared to the other tested genes.

5. A total of 14 VAGPs were presence among *S. suis* Thai isolates. However, the *SS2* carried only four VAGPs (*i. e.,* VAGP1 to VAGP4).

6. Dendrogram constructions based on RAPD patterns, VAGPs and STs were in complete agreement indicated that *SS2* isolates from Thailand could be divided into four groups and the majority (32/36) of isolates recovered from diseased pigs, slaughterhouse pigs and human patients could be classified into a single group (VAGP1, RAPD-A and ST1). This genetic information strongly suggests the transmission of *S. suis* isolates from pigs to humans in Thailand.

7. The non-*SS2* isolates in Thailand were highly diverse. However, a minority of non-*SS2* isolates (16 out of 209 isolates) carries VAGP1, VAGP2 and VAGP4 and related to *SS2* at a far distance suggesting that a rare strain of non-*SS2* carrying

human zoonotic transmission potential did exist and required carful monitor for the disease control.

8. r*SS2* ArcA was successfully produced and purified and inherit its original native enzymatic and biological properties. Analysis of r*SS2* ArcA deduced amino acid sequence indicated that it as a member of guanidine group-modifying enzymes superfamily which contains four conserved motifs (FTRD, EGGDV, MHLDT and CMSxP) and a catalytic triad, Cys-His-Glu/Asp.

9. Based on the results of pH dependent activity and acid tolerance of r*SS2* ArcA *in vitro*, it could be postulated that the *SS2* ArcA could functionally active throughout the phagocytosis mechanism. Moreover, these data supported that the *SS2* ArcA could facilitate *S. suis* survival in acidified environments.

10. An ArcA enzyme inhibition assay indicated that the *SS2* ArcA exhibits a considerably lower binding affinity to L-canavanine and L-NIO compared to its orthologous proteins, therefore, more study on structural information and kinetic are required to clarify the distinct binding behavior in order to define the reaction of L-canavanine and L-NIO inactions of *SS2* ArcA.

11. Partial inactivation of *SS2* ArcA by L-canavanine and L-NIO inhibitors were found to significantly reduce HE06 *SS2* survival in acidified DMEM medium as compared to the survival in acidified medium without inhibitors.

12. Data in this thesis indicated that it is worth testing further the involvement of *SS2* ArcA in facilitating the *SS2* survival in macrophage cells *in vitro* and in *S. suis* mouse infection model.

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APPENDICES

APPENDIX A

BACTERIAL CULTURE MEDIA

1. For recombinant *E. coli* clone

1.1 Luria-Bertani (LB) broth

LB broth composed of 10 g/L of tryptone (BD, MD 21152, United States), 5 g/L of yeast extract (Lab M Limited, Lancashire, UK), and 5 g/L of NaCl (Merck, Darmstadt, Germany). After dissolving completely all of these components in DW by stirring, it was sterilized by autoclaving and kept at 4 ºC.

1.2 LB agar

LB agar was prepared similarly to LB broth but included 15 g/L agar (Lab M Limited). After autoclaving and cooling down to 55–60 ºC, this medium was poured ~25 ml into each 100-mm petri dish (Biomed Co., Bangkok, Thailand). LB agar plates were stored at 4 ºC.

1.3 Antibiotics

LB broth or LB agar with antibiotics was prepared as described above. Antibiotics were added to broth and solid media at the following concentrations: 100 µg/ml of ampicililin (General Drug House, Bangkok, Thailand), 25 µg/ml of kanamycin (General Drug House), 50 µg/ml of spectinomycin (United States Biological, Massachusetts, USA), unless otherwise specified.

2. For *S. suis*

2.1 Blood agar

Blood agar composed of 40 g of Columbia blood agar base (Lab M Limited) in 1 l of UDW. After dissolving completely by stirring, it was sterilized by autoclaving. Before adding 5% human packed red cells, this medium was cooled down to 50 ºC for 2 h in water bath.

2.2 Todd-Hewitt broth (THB)

THB composed of 40 g of Todd-Hewitt powder (BD) in 1 l of UDW. The medium was sterilized by autoclaving.

2.3 Todd–Hewitt broth supplemented with 2% yeast extract (THY)

THY composed of 40 g of Todd-Hewitt powder (BD) and 20 g of Bacto yeast extract (BD) in 1 l of UDW. The medium was sterilized by autoclaving.

2.4 Todd-Hewitt agar (THA)

THA composed of 40 g of Todd-Hewitt powder and 15 g of agar in 1 l of UDW. The medium was sterilized by autoclaving. After autoclaving and cooling down to 55–60 ºC, this medium was poured ~25 ml into each 100-mm petri dish (Biomed Co., Bangkok, Thailand). THA plates were stored at 4 ºC.

2.5 Antibiotics

Blood agar, THB/THY or Todd-Hewitt agar with antibiotics was prepared as describe above. Antibiotics were added after sterilization by autoclaving at the following concentrations: 7.5 µg/ml of chloramphenicol (Sigma) and 250 µg/ml of spectinomycin (United States Biological), unless otherwise specified.

APPENDIX B

REAGENTS FOR PLASMID PREPARATION BY ALKALINE LYSIS METHOD

1. Solution-I (Plasmid Extraction buffer: 25 mM Tris-HCl, pH 8.0; 50 mM glucose; 10 mM EDTA)

The solution was prepared by dissolving the following ingredients that included of 0.90 g of glucose, 0.33 g of Tris (Ameresco, Ohio, USA) and 0.37 g of EDTA in 80 ml of DW. After dissolving completely, the pH was adjusted to 8.0 with 1 N HCl or 1 N NaOH and the volume was brought to 100 ml with UDW before autoclaving. The solution was stored at room temperature. One hundred ug/ml of RNase A was added in this solution before using.

2. Solution-II (Alkaline lysis buffer: 0.2 N NaOH, 1% (w/v) of SDS)

The solution was freshly prepared by mixing the ingredients together that composed of 0.20 ml of 5 N NaOH (Merck), 0.50 ml of 10% SDS (USB, Ohio, USA) and 4.30 ml of UDW.

3. Solution-III (3 M potassium acetate, pH 4.8)

The buffer was prepared by dissolving 2.44 g of potassium acetate (Univar, New South Wales, Australia) in 70 ml UDW. The pH of the solution was adjusted to 4.8 with glacial acetic acid (Merck). The volume was brought to 100 ml with UDW before autoclaving. The solution was stored at room temperature.

APPENDIX C

REAGENTS FOR DNA MANIPULATION AND ELECTROPHORESIS

1. 10x DNA loading dye

10x DNA loading dye included 5 mg of bromophenol blue (Bio-Rad laboratories), 5 mg of xylene cyanol FF (USB), 5 ml of glycerol (Merck) and 5 ml of 1x TE buffer, pH 8. It was kept at –20 °C. The final concentration of this dye is 1x when mixing with DNA sample prior to loading onto an agarose gel.

2. 1x Tris-EDTA (TE) buffer, pH 8

1x TE buffer composes of preparing two stock solutions that are 1 M Tris (Ameresco, Ohio, USA), pH 8 and 0.5 M EDTA, pH 8 (Merck). These solutions are mixed together based on desired volume. It was sterilized by autoclaving and stored at room temperature.

3. Ethidium bromide solution

The stock solution was prepared by dissolving 100 mg of ethidium bromide (Sigma) in 10 ml of UDW (Final concentration 10 mg/ml). This solution was kept at 4 °C and protected from light. For working solution, 50 µl of the stock solution was then added to 100 ml of DW to make 0.5 µg/ml working concentration.

4. 5x TBE buffer, pH 8.3

5x TBE buffer was prepared by dissolving 52.0 g of Tris (Ameresco, Ohio, USA), 27.5 g of boric acid (USB) and 4.65 g of EDTA·$2H_2O$ (Merck) in 700 ml of UDW. The pH of this solution was adjusted to 8.3 with concentrate HCl (Merck) and next adjusted the volume to 1 l with UDW. It was sterilized by autoclaving. For working solution, 5x TBE buffer was adjusted to 0.5x by DW and it can be reused three times.

5. GeneRuler™ DNA Ladder

83 ng/µl of each 1 kb DNA ladder and 100 bp plus DNA ladder (Thermo

Scientific) was prepared by diluting 33 µl of 0.5 µg/µl GeneRuler ^{TM} DNA Ladder stock solutions in 167 µl of 6x loading dye. It was kept at 4 ºC.

APPENDIX D

REAGENTS FOR RECOMBINANT PROTEIN PURIFICATION USING NI-NTA™ BEAD UNDER NATIVE PURIFICATION CONDITION

1. Phosphate buffer (1 M PB, pH 7.4 or 8.0)

The solution was conducted by preparing two stock solutions which composed of

Solution A

$Na_2HPO_4 \cdot 2H_2O$ (Merck) 177.90 g

DW 1,000 ml

Solution B

$NaH_2PO_4 \cdot 2H_2O$ (Univar) 156.01g

DW 1,000 ml

The solution B was slowly added to solution A for adjusting the pH to 7.4 or 8.0 and further sterilized by autoclaving.

2. 5 M Sodium chloride (NaCl) solution

292.20 g of NaCl (Merck) were dissolved in 1,000 ml of DW and they were sterilized using autoclaving.

3. 2x PBS extraction buffer, pH 7.4

The buffer contained the following ingredients: 20 ml of 1 M PB; pH 7.4, 60 ml of 5 M NaCl solution and 920 ml of UDW. This mixture was supplemented with 20% glycerol (Merck) and 5 mM of dithiothreitol (DTT) (Sigma).

4. Washing buffer (2x PBS extraction buffer, pH 8.0)

The buffer contained the following ingredients mixed together: 20 ml of 1 M PB, pH 8.0, 60 ml of 5 M NaCl solution and 920 ml of DW. This mixture was supplemented with 20% glycerol, 5 mM DTT (Sigma) and 0.5% Tween-20 (Sigma).

5. EDTA elution buffer

0.25 M EDTA (Merck) (9.306 g) was dissolved in 100 ml of UDW for stock solution. After dissolving, the solution was sterilized by autoclaving. Each stepwise of EDTA elution buffer containing 0.1, 0.25, 0.5 and 1.5 mM was prepared by diluting from 0.25 M EDTA in 2x PBS extraction buffer, pH 7.4 supplemented with 20% glycerol and 5 mM of DTT (Sigma).

Appendix E Orthologous proteins of peptide sequences generated from purified r*SS2* ArcA by LC/MS-MS and database search

	Reference					P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)	
	Scan(s)	Peptide	MH+	ΔM	z	P (pep)	XC	ΔCn	Sp	RSp	Ions	Count
1	gi|223933313|ref|ZP_03625302.1| arginine deiminase [*S. suis* 89/1591]					5.17E-06	250.22	40.83	46325.5	223933313	129 (106 17 4 2 0)	
	3250	R.CGGGDM*VAAGR.E	1067.15274	0.78126	2	2.67E-05	3.51	0.32	1622.2	1	19/20	5
	3254	R.CGGGDM*VAAGR.E	1067.15274	-0.92369	2	9.52E-06	3.70	0.37	2036.4	1	19/20	5
	4162	R.TDAASIEK.L	834.89459	-0.60792	2	9.93E-04	2.28	0.03	633.8	47	12/14	37
	4170	R.TDAASIEK.L	834.89459	-0.25972	2	1.02E-03	2.29	0.13	711.8	28	13/14	37
	4266	R.TDAASIEK.L	834.89459	-0.54163	1	2.08E-01	1.86	0.04	452.7	2	11/14	37
	4724	R.TDAASIEK.L	834.89459	-0.60731	2	8.37E-03	2.38	0.12	347.2	127	9/14	37
	4764	R.TDAASIEK.L	834.89459	-0.41438	2	5.73E-02	2.16	0.13	501.3	137	11/14	37
	4890	R.NRETLYGK.Y	981.08777	-0.41743	2	1.10E-02	2.69	0.08	696.6	9	11/14	35
	4992	R.CGGGDMVAAGR.E	1051.15332	-0.17537	2	8.91E-04	3.45	0.23	2142.5	1	19/20	5
	4998	K.VMLHRPGK.E	938.17590	-0.56923	2	2.19E-02	2.17	0.13	692.1	11	11/14	26
	5000	R.CGGGDMVAAGR.E	1051.15332	-0.16975	2	8.67E-03	3.89	0.20	2205.6	1	19/20	5
	5194	R.ETLYGK.Y	710.79828	-0.48877	1	3.78E-02	1.67	0.13	209.2	4	8/10	38
	5204	R.ETLYGK.Y	710.79828	-0.72339	1	1.20E-01	1.66	0.15	203.8	3	8/10	38
	5464	R.CM*SM*PFER.E	1090.24969	0.60998	2	5.54E-01	2.15	0.07	449.4	140	11/14	34
	5468	R.CM*SM*PFER.E	1090.24969	-0.34681	2	1.05E-01	2.05	0.07	494.0	25	11/14	34
	5520	R.NRETLYGK.Y	981.08777	0.42192	2	8.32E-04	2.29	0.04	624.5	13	11/14	35
	5828	K.IGGSELVR.G	830.95233	-0.45343	1	6.50E-01	1.64	0.08	202.4	56	9/14	5
	5886 - 5888	K.YIFTHHPEYGGK.V	1449.59412	0.03331	3	7.27E-06	2.69	0.06	584.8	7	22/44	10

Appendix E Orthologous proteins of peptide sequences generated from purified r*SS2* ArcA by LC/MS-MS and database search (Cont.)

Reference					P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)	
Scan(s)	Peptide	MH+	ΔM	z	P (pep)	XC	ΔCn	Sp	RSp	Ions	Count
5906	K.VPLVYNREETTR.I	1477.64734	-0.21748	2	9.11E-03	2.74	0.08	655.8	1	15/22	5
5940	K.VPLVYNR.E	861.02325	-0.77577	2	6.71E-03	2.18	0.06	551.6	2	11/12	6
5962	K.VPLVYNREETTR.I	1477.64734	-0.11116	2	1.12E-03	3.24	0.16	527.2	7	13/22	5
6006	R.CMSM*PFER.E	1074.25029	0.09546	2	1.67E-01	2.19	0.02	582.5	3	12/14	34
6012	R.CMSM*PFER.E	1074.25029	0.19629	2	1.14E-01	2.32	0.12	464.1	8	11/14	34
6018	K.YIFTHHPEYGGK.V	1449.59412	-0.82088	2	9.10E-05	3.93	0.33	1360.0	1	17/22	10
6044	K.IGGSELVR.G	830.95233	-0.46144	2	3.48E-03	2.65	0.11	906.9	1	13/14	5
6052	K.VPLVYNR.E	861.02325	-0.57623	1	5.46E-03	2.10	0.23	143.1	18	8/12	6
6054	K.YIFTHHPEYGGK.V	1449.59412	-0.54194	2	6.63E-06	3.53	0.24	1543.2	1	18/22	10
6068	K.VPLVYNR.E	861.02325	-0.54303	1	2.65E-02	1.88	0.22	165.5	7	9/12	6
6118	K.IGGSELVR.G	830.95233	-0.05421	2	1.04E-03	2.59	0.11	1024.3	1	13/14	5
6306	K.VPLVYNR.E	861.02325	-0.62501	2	9.95E-02	2.27	0.17	604.3	10	11/12	6
6308 - 6310	K.YIFTHHPEYGGK.V	1449.59412	0.31557	3	1.27E-03	2.73	0.07	865.6	1	26/44	10
6328	R.IEGGDELVLSK.D	1160.29956	1.89336	2	9.88E-06	3.53	0.28	784.8	2	17/20	33
6364	K.VPLVYNR.E	861.02325	-0.56349	2	2.13E-03	2.09	0.08	635.4	1	11/12	6
6372	R.IEGGDELVLSK.D	1160.29956	1.51055	2	4.08E-05	3.39	0.24	825.1	1	18/20	33
6398	K.DVLAVGISQR.T	1058.21362	-0.32088	2	2.79E-04	3.31	0.15	1679.2	1	17/18	34
6402	K.DVLAVGISQR.T	1058.21362	-0.54439	2	9.09E-05	3.56	0.17	1576.9	1	17/18	34
6504	K.DVLAVGISQR.T	1058.21362	-0.67273	1	6.21E-03	2.65	0.10	282.5	11	12/18	34
6556 - 6558	R.EETTRIEGGDELVLSK.D	1776.92358	-0.55822	3	1.99E-04	2.91	0.03	909.5	4	23/60	9

Appendix E Orthologous proteins of peptide sequences generated from purified r*SS2* ArcA by LC/MS-MS and database search (Cont.)

Reference					P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)	
Scan(s)	Peptide	MH+	ΔM	z	P (pep)	XC	ΔCn	Sp	RSp	Ions	Count
6570	K.DVLAVGISQR.T	1058.21362	-0.58582	1	2.91E-03	2.67	0.05	341.6	38	12/18	34
6600	K.DVLAVGISQR.T	1058.21362	1.55066	1	3.65E-02	2.86	0.06	323.5	3	12/18	34
6660	K.RNTITNAILESK.G	1360.54187	-0.01668	2	3.99E-02	2.98	0.11	710.3	3	15/22	4
6662	K.EIENLM*PDYLER.L	1538.70455	-1.12553	2	1.42E-04	4.30	0.23	937.6	1	17/22	16
6668	K.EIENLM*PDYLER.L	1538.70455	0.20077	2	1.17E-04	3.94	0.08	872.1	2	17/22	16
6680	K.VPLVYNR.E	861.02325	-0.11842	2	2.12E-01	2.04	0.12	648.4	2	11/12	6
6682	R.IEGGDELVLSK.D	1160.29956	0.62236	2	3.49E-03	3.16	0.18	835.3	1	18/20	33
6686	R.IEGGDELVLSK.D	1160.29956	0.68523	2	1.28E-04	3.35	0.22	782.0	1	17/20	33
6692	K.FTIHPEIEGDLR.V	1427.58679	-0.05337	3	2.44E-03	3.70	0.10	1436.1	1	26/44	21
6762	K.KVLAFEFANNRK.F	1437.67114	0.79074	3	2.20E-04	3.17	0.06	654.9	2	22/44	34
6806	K.FTIHPEIEGDLR.V	1427.58679	-0.55244	2	1.13E-05	2.78	0.27	566.3	8	14/22	21
6810	K.FTIHPEIEGDLR.V	1427.58679	-0.12556	2	1.05E-05	3.12	0.24	868.5	1	17/22	21
6856	K.VLAFEFANNRK.F	1309.49829	1.29790	2	5.99E-05	3.05	0.07	1100.7	3	16/20	34
6856	K.KVLAFEFANNR.K	1309.49829	1.29790	2	5.76E-02	2.60	0.19	795.1	12	15/20	34
6860	K.VLAFEFANNRK.F	1309.49829	-0.45967	2	5.17E-06	2.90	0.05	864.4	2	15/20	34
6860	K.KVLAFEFANNR.K	1309.49829	-0.45967	2	9.97E-01	2.30	0.21	518.1	68	13/20	34
6862	K.DVLAVGISQR.T	1058.21362	-1.24470	2	2.71E-03	2.43	0.03	797.3	4	15/18	34
6862	R.CMSMPFER.E	1058.25085	-1.28193	2	9.19E-01	2.35	0.12	792.0	5	12/14	34
6876	K.DVLAVGISQR.T	1058.21362	-0.74910	2	3.00E-04	2.87	0.13	969.1	1	16/18	34
6876	R.CMSMPFER.E	1058.25085	-0.78633	2	9.18E-02	2.49	0.15	765.2	6	12/14	34

Appendix E Orthologous proteins of peptide sequences generated from purified r*SS2* ArcA by LC/MS-MS and database search (Cont.)

Reference					P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)	
Scan(s)	Peptide	MH+	ΔM	z	P (pep)	XC	ΔCn	Sp	RSp	Ions	Count
6900	R.NTITNAILESK.G	1204.35559	-0.63135	1	9.98E-01	2.43	0.10	336.8	159	12/20	18
6940 - 6942	K.EIENLM*PDYLER.L	1538.70455	-0.63273	2	2.00E-04	4.27	0.16	699.7	1	16/22	16
6958	R.IEGGDELVLSK.D	1160.29956	0.66936	2	6.19E-01	2.71	0.11	718.0	1	17/20	33
6976 - 6978	K.FTIHPEIEGDLR.V	1427.58679	-0.65011	3	3.21E-03	3.71	0.12	1329.6	2	25/44	21
6984	R.IEGGDELVLSK.D	1160.29956	-1.00374	2	1.42E-03	2.92	0.20	693.6	1	16/20	33
6998	K.LAAESLVTPEIR.E	1299.49841	-1.04329	2	6.36E-06	3.90	0.12	1054.7	2	17/22	4
7000 - 7002	K.VLAFEFANNRK.F	1309.49829	0.72614	3	7.24E-03	2.56	0.13	329.5	8	18/40	34
7022	R.DEGVEVLYLEK.L	1294.43225	-0.39900	2	3.55E-04	3.95	0.33	1344.2	1	17/20	4
7030	R.DEGVEVLYLEK.L	1294.43225	0.47710	2	6.13E-05	4.15	0.26	1459.1	1	17/20	4
7110	K.VPLVYNR.E	861.02325	0.18120	2	1.02E-01	2.08	0.07	480.2	2	10/12	6
7136	K.VLAFEFANNR.K	1181.32532	1.33855	2	9.68E-06	3.81	0.14	1842.9	1	17/18	34
7156	K.VLAFEFANNR.K	1181.32532	1.71001	2	9.62E-05	3.70	0.17	1521.7	1	16/18	34
7168	K.DVLAVGISQR.T	1058.21362	0.17522	2	6.36E-04	3.17	0.14	1117.3	2	14/18	34
7184	K.DVLAVGISQR.T	1058.21362	1.28191	2	1.00E-03	3.35	0.12	1499.5	1	16/18	34
7228	K.VLAFEFANNR.K	1181.32532	-1.94099	2	1.69E-04	3.11	0.36	1918.1	1	17/18	34
7264	K.LAAESLVTPEIR.E	1299.49841	0.27764	2	4.90E-02	3.48	0.16	906.5	36	15/22	4
7272	K.LAAESLVTPEIR.E	1299.49841	0.63310	2	1.14E-04	3.55	0.15	794.4	29	15/22	4
7276	K.VLAFEFANNRK.F	1309.49829	-0.58357	2	1.38E-03	2.91	0.07	1280.8	1	16/20	34
7294	R.IEGGDELVLSK.D	1160.29956	-0.66267	2	3.87E-02	3.11	0.10	702.5	4	16/20	33
7306	K.FTIHPEIEGDLR.V	1427.58679	0.45145	3	3.04E-02	2.70	0.15	422.3	51	17/44	21

Appendix E Orthologous proteins of peptide sequences generated from purified r*SS2* ArcA by LC/MS-MS and database search (Cont.)

Reference					P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)	
Scan(s)	Peptide	MH+	ΔM	z	P (pep)	XC	ΔCn	Sp	RSp	Ions	Count
7318	R.NTITNAILESK.G	1204.35559	-0.87117	2	6.02E-03	2.66	0.01	896.1	1	15/20	18
7326	R.IEGGDELVLSK.D	1160.29956	-1.02339	2	4.04E-01	2.17	0.18	461.2	3	13/20	33
7330	R.DEGVEVLYLEK.L	1294.43225	-0.21699	2	2.72E-03	3.45	0.21	1132.9	1	16/20	4
7334	K.VLAFEFANNRK.F	1309.49829	-0.51192	2	1.98E-03	2.89	0.13	928.1	1	15/20	34
7336	R.DEGVEVLYLEK.L	1294.43225	-0.71492	2	9.44E-05	3.51	0.29	1046.2	2	15/20	4
7458	K.EIENLMPDYLER.L	1522.70520	0.96172	2	3.24E-05	4.09	0.20	986.8	1	18/22	16
7482	K.YIFTHHPEYGGK.V	1449.59412	0.14780	3	6.50E-03	2.73	0.06	525.7	1	21/44	10
7484	K.DVLAVGISQR.T	1058.21362	-0.62813	2	5.62E-05	3.61	0.15	1622.6	1	16/18	34
7506	K.DVLAVGISQR.T	1058.21362	-0.38594	2	1.46E-04	3.51	0.13	1281.5	1	16/18	34
7556	K.VLAFEFANNR.K	1181.32532	-0.24751	2	4.27E-03	3.14	0.07	1304.9	1	16/18	34
7576	K.LAAESLVTPEIR.E	1299.49841	0.09709	2	6.41E-05	3.41	0.18	1117.4	9	16/22	4
7612	K.VLAFEFANNR.K	1181.32532	-1.23274	2	9.68E-05	3.11	0.09	674.4	1	14/18	34
7632	K.YIFTHHPEYGGK.V	1449.59412	0.20989	2	4.36E-03	3.11	0.03	688.7	1	16/22	10
7668	R.IEGGDELVLSK.D	1160.29956	-0.33296	2	1.66E-02	2.56	0.12	409.1	52	13/20	33
7812	K.DVLAVGISQR.T	1058.21362	-0.96504	2	1.23E-04	3.05	0.16	1499.1	1	16/18	34
7834	K.DVLAVGISQR.T	1058.21362	-0.45210	2	1.94E-04	3.53	0.14	1654.1	1	16/18	34
7846	K.IGGSELVR.G	830.95233	1.30297	2	5.39E-02	2.04	0.11	890.9	1	13/14	5
7854	K.IGGSELVR.G	830.95233	-0.72780	2	1.80E-01	2.07	0.12	712.0	2	13/14	5
7858	K.LAAESLVTPEIR.E	1299.49841	-0.08674	2	3.17E-02	3.31	0.14	763.1	22	14/22	4
7866	K.LAAESLVTPEIR.E	1299.49841	-0.81843	2	4.06E-01	2.40	0.06	420.4	89	13/22	4

Appendix E Orthologous proteins of peptide sequences generated from purified r*SS2* ArcA by LC/MS-MS and database search (Cont.)

Reference					P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)	
Scan(s)	Peptide	MH+	ΔM	z	P (pep)	XC	ΔCn	Sp	RSp	Ions	Count
7900	K.VLAFEFANNR.K	1181.32532	-0.46223	2	1.37E-02	2.08	0.05	456.6	11	12/18	34
7920	K.FTIHPEIEGDLR.V	1427.58679	-0.04925	3	2.10E-03	3.02	0.14	455.9	1	20/44	21
7968	R.IEGGDELVLSK.D	1160.29956	0.80303	2	2.73E-02	2.95	0.27	636.5	1	15/20	33
8122	K.LAAESLVTPEIR.E	1299.49841	-0.49141	2	6.27E-01	2.43	0.05	605.8	57	15/22	4
8126	K.DVLAVGISQR.T	1058.21362	-0.65376	2	1.40E-03	3.57	0.09	1674.5	1	17/18	34
8134	K.LAAESLVTPEIR.E	1299.49841	0.79033	2	2.84E-02	3.03	0.09	740.7	31	15/22	4
8138	K.DVLAVGISQR.T	1058.21362	-1.99055	2	3.44E-03	2.69	0.16	735.9	1	15/18	34
8178	K.DVLAVGISQR.T	1058.21362	1.19914	2	9.94E-05	3.66	0.12	1965.4	1	17/18	34
8196	R.NTITNAILESK.G	1204.35559	-0.65144	2	1.00E-01	2.03	0.12	178.0	184	11/20	18
8208	K.FTIHPEIEGDLR.V	1427.58679	-1.12912	3	3.43E-02	2.81	0.20	401.4	2	19/44	21
8218	K.DVLAVGISQR.T	1058.21362	1.39092	2	1.14E-04	3.53	0.11	1650.3	1	16/18	34
8228	R.IEGGDELVLSK.D	1160.29956	-0.41328	2	2.62E-01	2.21	0.09	514.7	4	13/20	33
8240	K.VLAFEFANNR.K	1181.32532	-1.25178	2	6.17E-02	2.33	0.09	379.3	8	13/18	34
8274	K.VLAFEFANNR.K	1181.32532	1.82292	2	6.86E-04	2.82	0.19	531.2	4	12/18	34
8324	R.DEGVEVLYLEK.L	1294.43225	-0.28108	2	6.84E-02	2.35	0.23	333.0	34	12/20	4
8342	R.IEGGDELVLSK.D	1160.29956	0.78618	2	7.66E-03	2.77	0.18	672.9	1	15/20	33
8350	R.IEGGDELVLSK.D	1160.29956	0.92278	2	1.09E-02	3.04	0.12	528.3	21	15/20	33
8384	K.IGGSELVR.G	830.95233	1.19249	2	5.71E-02	2.08	0.03	648.0	7	12/14	5
8430	K.LAAESLVTPEIR.E	1299.49841	-0.13765	2	3.99E-03	3.68	0.14	1609.2	1	18/22	4
8452	K.LAAESLVTPEIR.E	1299.49841	-0.88338	2	7.88E-04	2.70	0.22	660.9	15	14/22	4

Appendix E Orthologous proteins of peptide sequences generated from purified r*SS2* ArcA by LC/MS-MS and database search (Cont.)

Reference					P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)	
Scan(s)	Peptide	MH+	ΔM	z	P (pep)	XC	ΔCn	Sp	RSp	Ions	Count
8488	K.DVLAVGISQR.T	1058.21362	-0.37959	2	8.93E-05	3.26	0.12	1313.0	1	15/18	34
8492	K.DVLAVGISQR.T	1058.21362	-0.50313	2	9.92E-05	3.53	0.18	1577.7	1	17/18	34
8534	R.DEGVEVLYLEK.L	1294.43225	-0.45369	2	1.07E-03	2.33	0.25	336.3	1	13/20	4
8564 - 8566	K.VLAFEFANNRK.F	1309.49829	-0.00673	2	4.54E-04	2.82	0.19	554.6	7	14/20	34
8568 - 8570	K.VLAFEFANNR.K	1181.32532	0.18688	2	6.31E-04	2.82	0.22	628.2	2	15/18	34
8694	R.IEGGDELVLSK.D	1160.29956	0.88127	2	1.06E-02	2.04	0.09	374.6	155	13/20	33
8746	K.DVLAVGISQR.T	1058.21362	-1.48213	2	1.11E-03	2.68	0.13	844.7	1	16/18	34
8780	K.DVLAVGISQR.T	1058.21362	-0.41316	2	2.33E-03	3.57	0.13	1006.5	1	15/18	34
8838	R.NTITNAILESK.G	1204.35559	0.95354	2	7.19E-02	2.34	0.09	363.8	3	13/20	18
8862	K.VLAFEFANNR.K	1181.32532	1.98564	2	2.07E-03	2.98	0.20	695.1	1	14/18	34
8958	K.VLAFEFANNR.K	1181.32532	1.77629	2	4.50E-02	2.66	0.04	690.3	1	15/18	34

APPENDIX F

ANIMAL ETHIC APPROVAL FROM THAMMASAT UNIVERSITY ANIMAL CARE AND USE COMMITTEE

ใบรับรองการอนุมัติให้ดำเนินการเลี้ยงและใช้สัตว์

รหัสโครงการ 3/2552

ชื่อข้อเสนอการวิจัย

(ภาษาไทย) การพัฒนาวัคซีนชนิด subunit ที่คัดเลือกจาก immunomic approach สำหรับป้องกันโรคติดเชื้อที่มีสาเหตุจากเชื้อแบคทีเรีย สเตร็บโตค็อกคัส ซูอิส ในสุกรและสัตว์

(ภาษาอังกฤษ) Development of subunit vaccine based on immunomic approach for *Streptococcus suis* infection in swine and other animals

ชื่อ-สกุล ผู้เสนอโครงการวิจัย ผู้ช่วยศาสตราจารย์ ดร.พจนีย์ ศรีมาโนชญ์

หน่วยงานสังกัด (คณะ/กอง) ชีวเวชศาสตร์

(มหาวิทยาลัย/กรม) มหาวิทยาลัยธรรมศาสตร์

(กระทรวง) กระทรวงศึกษาธิการ

ข้อเสนอการวิจัยนี้ได้ผ่านการพิจารณาจากคณะอนุกรรมการจริยธรรมการวิจัยในสัตว์ มหาวิทยาลัยธรรมศาสตร์แล้ว เห็นว่ามีความสอดคล้องกับจรรณยาบรรณการใช้สัตว์เพื่องานทางวิทยาศาสตร์ สภาวิจัยแห่งชาติ จึงเห็นสมควรให้ดำเนินการเลี้ยงและใช้สัตว์ ตามข้อเสนอการวิจัยนี้ได้

ลงนาม .....

(รองศาสตราจารย์ ดร.ไพโรค ปัญจะ)

ประธานคณะอนุกรรมการจริยธรรมการวิจัยในสัตว์

มหาวิทยาลัยธรรมศาสตร์

วัน/เดือน/ปี ๑๗ ก.ค. ๕๓

ลงนาม .....

(รองศาสตราจารย์ นายแพทย์ปรีชา วาณิชยเศรษฐกุล)

รองอธิการบดีฝ่ายวิจัย

ปฏิบัติราชการแทนอธิการบดี

วัน/เดือน/ปี 15 ม.ค. 53

APPENDIX G

REAGENTS FOR INDIRECT ELISA

1. Coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6)

The buffer was prepared by dissolving 2.93 g of $NaHCO_3$ and 0.53 g of Na_2CO_3 in 700 ml of DW. After completely dissolving, pH of the solution was adjusted to 9.6 with NaOH. The volume was then made up to 1 l with DW and further sterilized by autoclaving.

2. Phosphate buffer (1 M PB, pH 7.4)

The solution was conducted by preparing two stock solutions which composed of

Solution A

$Na_2HPO_4 \cdot 2H_2O$ (Merck) 177.90 g

DW 1,000 ml

Solution B

$NaH_2PO_4 \cdot 2H_2O$ (Univar) 156.01 g

DW 1,000 ml

The solution B was slowly added to solution A for adjusting the pH to 7.4 and further sterilized by autoclaving.

3. 3 M Sodium chloride (NaCl) solution

175.32 g of NaCl (Merck) was dissolved in 1,000 ml of DW and it was sterilized by autoclaving.

4. Phosphate buffered saline (0.01 M PBS, pH 7.4)

The buffer contained the following ingredients:

10 ml of 1 M PB, pH 7.4

50 ml of 3 M NaCl solution

940 ml of DW

5. Washing solution (PBS-T: 0.05% Tween-20 in 0.01 M PBS, pH 7.4)

The solution was prepared by adding 0.5 ml of Tween-20 (Sigma) with 1 l of PBS, pH 7.4.

6. Blocking solution

The solution was prepared by dissolving 1 g of bovine serum albumin (BSA), (Fraction V) (Sigma) or 5% fetal bovine serum in 100 ml of 0.01 M PBS, pH 7.4.

5. Diluent solution

This buffer was prepared by dissolving 0.2 g of BSA in 100 ml of PBS-T or using 0.2% fetal bovine serum in PBS-T. The solution was stored at 4 ºC until use.

6. Goat anti-mouse IgG secondary antibody, horseradish peroxidase conjugate

Goat anti-mouse IgG-horseradish peroxidase conjugate (KPL, USA) was diluted with diluent solution at 1:3,000.

7. 0.1 M citrate buffer, pH 4.5

29.40 g of trisodium citrate ($Na_3C_6H_5O_7 \cdot H_2O$) was dissolved in 600 ml of DW. The pH was adjusted to 5.2 with glacial acetic acid (Merck), and next adjusted the volume to 1,000 ml with DW. It was sterilize by autoclaving, and stored at 4 ºC until use.

8. Substrate solution for ELISA

The solution was freshly prepared by mixing the following ingredients together:

0.01 g of 1, 4-*p*-phenylene-diaminedihydrochloride (PPD) (Sigma)

0.01 ml of 30% H_2O_2

10 ml of 0.1 M citrate buffer, pH 4.5

APPENDIX H

REAGENTS FOR SDS-PAGE AND WESTERN BLOTTING AND STAINING OF PROTEIN IN THE GEL

1. Sample buffer (Non-reducing buffer)

This buffer was prepared as by combining the following ingredients: 3.65 ml of UDW, 1.25 ml of 0.5 M Tris-HCl, pH 6.8 , 2.5 ml of glycerol, 2.0 ml of 10% (w/v) SDS and 0.1 ml of 1% bromophenol blue. This buffer was kept in a 1 ml-aliquot at −20 °C until use. For SDS-PAGE, at least one parts of the sample was diluted with one part of sample buffer and next heated at 100 °C for 10 min. Heated sample was centrifuged at 12,000 *× g* for 5 min before loading onto gel.

2. 6x Sample buffer (Reducing buffer)

50 µl of ß-mercaptoethanol was added into 950 µl of sample buffer (Non-reducing buffer). It was kept in a 1 ml-aliquot at −20 °C until use. For SDS-PAGE, one parts of the sample was diluted with five parts of sample buffer and next heated at 100 °C for 10 min. Heated sample was centrifuged at 12,000 *× g* for 5 min before loading onto gel.

3. Tris-HCl (1.5 M, pH 8.8)

To prepare this solution, 18.15 g of Tris-base were dissolved in 50 ml of UDW, and then the pH was adjusted to 8.8 with 1 N HCl. The volume was brought up to 100 ml with UDW. The solution was filtered through a sterile 0.2 µm filter membrane and stored at 4 °C until use.

4. Tris-HCl (0.5 M, pH 6.8)

To prepare the solution, 6.05 g of Tris-base were dissolved in 50 ml of DW, then pH was adjusted to 6.8 with 1 N HCl. The volume was brought up to 100 ml with DW. The solution was filtered through a sterile 0.2 µm filter membrane and stored at 4 °C until use.

5. Sodium dodecyl sulfate solution (SDS, 10%)

The solution was prepared by dissolving 10 g of SDS in 100 ml of UDW. The solution was filtered through a Whatman filter paper.

6. Ammonium persulfate solution (10%)

The solution was freshly prepared by dissolving 50 mg of ammonium persulfate (Univar) in 0.5 ml of UDW.

7. Polyacrylamide resolving gel (12%)

The following solutions were combined with 3.4 ml of UDW:

2.5 ml of 1.5 M Tris-HCl, pH 8.8

0.1 ml of 10% SDS solution

4.0 ml of 30% acrylamide solution

The preparation was degassed under a vacuum for at least 5 minutes. Gel polymerization was initiated by adding 50 µl of 10% ammonium persulfate (Univar) and 5 µl of TEMED (Sigma). The preparation was poured into the gel casting apparatus, over-layered with UDW and allowed to polymerize at 25 ºC for at least 40 min before use.

8. Polyacrylamide stacking gel (4%)

The following solutions were combined with 3.05 ml of UDW:

1.25 ml of 0.5 M Tris-HCl, pH 6.8

0.05 ml of 10% SDS solution

0.65 ml of 30% acrylamide solution (Bio-Rad Laboratories)

The preparation was degassed under a vacuum for at least five min. Gel polymerization was initiated by adding 25 µl of 10% ammonium persulfate (Univar) and 5 µl of TEMED (Sigma). The preparation was gently layered onto the polymerized 12% resolving gel in the gel casting apparatus. A comb was properly placed and the gel was allowed to polymerize at 25 ºC for at least 30 minutes before use.

9. 10x electrode buffer for SDS-PAGE

The buffer contained the following ingredients:

30.3 g of Tris base

142.9 g of Glycine (USB)

10 g of SDS

The reagents were dissolved in UDW and the volume was made up to 1 l with UDW. This buffer was sterilized by autoclaving and then stored at 4 ºC. This stock buffer was diluted to 1x buffer before use.

10. Staining of the protein in the gel

The proteins in the polyacrylamide gel were fixed in freshly prepared buffer containing 1% *o*-phosphoric acid and 20% methanol in deionized water for 1 h. The gel was immediately immerged and kept overnight in dye solution (0.1% (w/v) Coomassie Brilliant Blue G-250 dye powder (Sigma), 8% ammonium sulfate, 1.9% *o*-phosphoric acid (Merck) and 20% methanol (Merck) in UDW). The stained gel was washed several times by soaking in deionized water until the background became clear. The gel with visualized proteins was either photographed or dried on cellophane membrane

11. Ponceau S solution

0.1 % (w/v) of Ponceau S ($C_{22}H_{12}N_4O_{13}S_4Na_4$) was mixed with 5% acetic acid (Merck). This colored solution ready-to-use and it rapidly stained of protein bands on nitrocellulose membranes. Ponceau S staining on membrane is reversible by washing the stained membrane with DW.

12. 5% blocking solution

The solution was prepared by dissolving 0.5 g of skim milk (Sigma) in 10 ml of PBS, pH 7.4.

13. Transfer buffer

The buffer contained the following ingredients:

6.06 g of Tris

28.80 g of Glycine (USB)

400 ml of Methanol

The reagents were dissolved in 1,600 ml of UDW and the volume was made up to 2 l with UDW. This buffer was stored at room temperature.

14. Washing buffer (PBS-T: 0.05% Tween-20 in 0.01 M PBS, pH 7.4)

The solution was prepared by adding 0.5 ml of Tween-20 (Sigma) to 1 l of PBS, pH 7.4

15. Diluent

This buffer was prepared by dissolving 0.2 ml of fetal bovine serum in 100 ml of PBS-T.

16. Tris buffer (0.15 M Tris-HCl, pH 9.6)

To prepare this buffer, 18.15 g of Tris were dissolved in 700 ml of UDW. After dissolving, the pH of the solution was adjusted to 9.6 with 1 N HCl and the volume was made up to 1 l with UDW.

APPENDIX I

REAGENTS FOR DETERMINING ENZYMATIC ACTIVITY OF ARGININE DEIMINASE

1. 1 M Potassium phosphate buffer, pH 7.2

The solution was conducted by preparing two stock solutions which composed of

1 M monobasic, anhydrous stock

136.09 g of KH_2PO_4 (Merck, Darmstadt, Germany)

1,000 ml of DW

1 M Dibasic, anhydrous stock

174.18 g of $K_2HPO_4 \cdot 2H_2O$ (Merck, Darmstadt, Germany)

1,000 ml of DW

The monobasic solution was slowly added to dibasic solution for adjusting the pH to 7.2 and further sterilized by autoclaving. The solution was stored at room temperature.

2. Assay buffer (2x PBS extraction buffer, pH 7.4)

The buffer contained the following ingredients:

20 ml of 1 M PB, pH 7.4

60 ml of 5 M NaCl solution

920 ml of DW

3. 0.5 M L-arginine in 100 mM Potassium phosphate buffer, pH 7.2

To prepare this buffer, 0.871 g of L-arginine were dissolved in 10 ml of 100 mM potassium phosphate buffer, pH 7.2. After dissolving, the solution was filtered through 0.2 µm filter membrane and stored at -20 ºC until use.

4. 1:3 mixture (v/v) of 95% H_2SO_4 : 85% H_3PO_4

62.5 ml of 95% H_2SO_4 (Merck) were mixed with 187.5 ml of 85% H_3PO_4

(Merck). This solution was kept at 25 °C and protected from light.

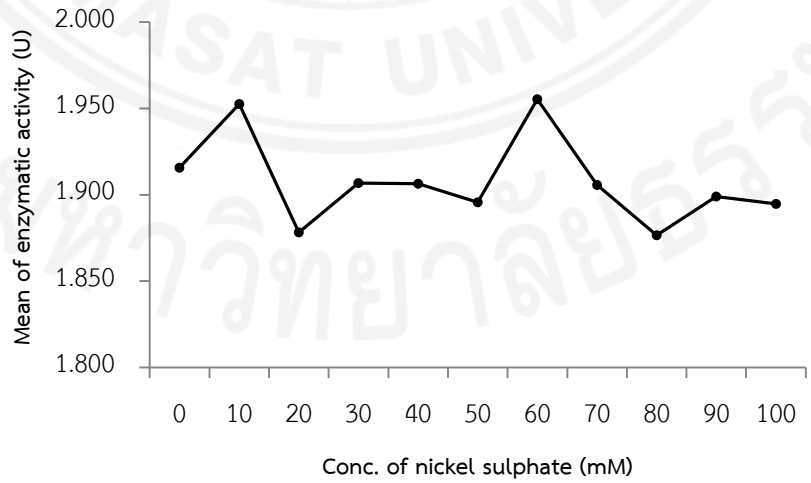
5. 3% diacetyl monoxime

The solution was freshly prepared by dissolving 0.3 g of diacetyl monoxime (2, 3-Butanedione monoxime) (Sigma) in 10 ml of UDW. It was kept at room temperature under light protection.

APPENDIX J

DETERMINATION OF THE EFFECT OF NICKEL SULFATE TO ENZYMATIC ACTIVITY IN LYSATE OF RECOMBINANT *SS2* ARGININE DEIMINASE TRANSFORMANT E. COLI

Conc. of nickel sulfate (mM)	Mean of enzymatic activity (U)	%
0	1.916	100.00
10	1.953	101.92
20	1.878	98.05
30	1.907	99.54
40	1.906	99.51
50	1.896	98.95
60	1.955	102.07
70	1.906	99.47
80	1.877	97.96
90	1.899	99.13
100	1.895	98.90



The enzymatic activity of lysate of r*SS2* ArcA transformant *E. coli* clone after reacted with each concentration of nickel sulfate

APPENDIX K

REAGENTS FOR PREPARATION OF ELECTRO-COMPETENT S. SUIS CELLS

1. Chemical transformation buffer (CTB)

The CTB composed of 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl and 10 mM pipes (piperazine-N, N′-bis (2-ethanesulfonic acid), pH 6.7. Each solution was filtered through a sterile 0.2 µm filter membrane and stored at 4 ºC until use.

2. Electroporation buffer (EB)

The EB was specifically designed for *S. suis* that included of 0.3 M sucrose and 2 mM potassium phosphate, pH 8.4. Each solution was filtered through a sterile 0.2 µm filter membrane and stored at 4 ºC until use.

BIOGRAPHY

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Scholarship	2008: Thailand Graduate Institute of Science and Technology (TGIST; TG-22-13-51-008D), NSTDA

Publications

1. **Maneerat K**, Yongkiettrakul S, Kramomtong I, Tongtawe P, Tapchaisri P, Luangsuk P, et al. Virulence genes and genetic diversity of *Streptococcus suis* serotype 2 isolates from Thailand. Transbound Emerg Dis. 2013 Nov;60 Suppl 2:69–79.
2. **Maneerat K**, Yongkiettrakul S, Jiemsup S, Tongtawe P, Gottschalk M, Srimanote P. Expression and characterization of serotype 2 *Streptococcus suis* arginine deiminase. (Submitted to Journal of Microbiological Methods)

Oral presentations

International conferences

1. **Maneerat K**, Yongkiettrakul S, Kramomtong I, Tongtawe P, Tapchaisri P, Luangsuk P, et al. Virulence genes of serotype 2 *Streptococcus suis* Thai isolates and their genetic diversity. Joint conference on emerging and re-emerging epidemics affecting global health 2012, September 19th to 22nd, 2012, Orvieto, Italy. (**Travelling scholarship; 2012 The Alain Provost award**)

2. **Maneerat K**, Yongkiettrakul S, Tongtawe P, Tapchaisri P, Gottschalk M, Srimanote P. Characterization of arginine deiminase of serotype 2 *Streptococcus suis*. The 1st International Conference on Medical Innovation for Health, November 4th to 6th, 2014, Rama Gardens Hotel, Bangkok, Thailand. (**Consolation prize**)

National conferences

1. Maneerat K. Genetic diversity of *Streptococcus suis* Thai isolates. กิจกรรมการนำเสนอผลงานปริญญานิพนธ์และวิทยานิพนธ์ นักศึกษาทุน TGIST NUI-RC YSTP และ ผนวท เมื่อวันที่ 27 เมษายน 2555 ณ ศูนย์ประชุมอุทยานวิทยาศาสตร์ประเทศไทย, สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ (สวทช.)

2. **Maneerat K,** Yongkiettrakul S, Kramomtong I, Tongtawe P, Tapchaisri P, Luangsuk P, et al. Virulence genes and genetic diversity of *Streptococcus suis* serotype 2 isolates from Thailand. 2nd Joint symposium of Thammasat University and BK12 PlUS of CUK, January 21st to 23rd, 2015, Faculty of Allied Health Sciences, Thammasat University (Rangsit Campus), Thailand.

Poster presentations

International conference

1. **Maneerat K**, Yongkiettrakul S, Tongtawe P, Tapchaisri P, Chaicumpa W, Gottschalk M, et al. Characterization of arginine deiminase of serotype 2 *Streptococcus suis*. XIX Lancefield International Symposium on Streptococci and Streptococcal Diseases**,** November 9th to 12th, 2014, Buenos Aires, Argentina. (Electronic poster)

National conferences

1. **Maneerat K**, Yongkiettrakul S, Kramomtong I, Tongtawe P, Tapchaisri P, Luangsuk P, et al. Distribution of virulence genes of *Streptococcus suis* isolates in Thailand and their random amplified polymorphic DNA patterns. The national conference of zoonotic diseases, July 15th, 2010, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

2. Srimanote P, **Maneerat K**, Sakolvaree Y, Chaicumpa W. Identification of diagnostic marker and vaccine candidate for *Streptococcus suis* infection. งานประชุม "นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว" เมื่อวันที่ 14–16 ตุลาคม 2553 ณ โรงแรมฮอลิเดย์ อินน์ รีสอร์ท รีเจนท์ บีช ชะอำ จังหวัดเพชรบุรี (**รางวัลโปสเตอร์ดีเยี่ยม สาขาวิทยาศาสตร์การแพทย์**)

3. **Maneerat K**, Yongkiettrakul S, Kramomtong I, Tongtawe P, Tapchaisri P, Luangsuk P, et al. Distribution of virulence genes of *Streptococcus suis* isolates in Thailand and their random amplified polymorphic DNA patterns. The fourth Scientific Meeting in Allied Health Sciences, February 21st, 2011, Faculty of Allied Health Sciences, Thammasat University (Rangsit Campus), Pathum Thani, Thailand.

Conferences attended:

1. The Second Scientific Meeting in Allied Health Sciences: New Approach for Research on Emerging and Re-emerging Diseases of Thailand, February 20th to 21st, 2009, Asian Institute of Technology and Thammasat University (Rangsit Campus), Pathum Thani, Thailand.

2. The Third Scientific Meeting in Allied Health Sciences, February 19th, 2010, Faculty of Allied Health Sciences, Thammasat University (Rangsit Campus), Pathum Thani, Thailand.

3. The 32nd Phamacological and Therapeutic Society of Thailand Meeting, March 26th, 2010, Architecture and Planning Auditorium, Thammasat University (Rangsit Campus), Pathum Thani, Thailand.

4. First Thai National Symposium on Animal Care & Use for Scientific Purpose (1st TACUS) & Laboratory Animal Trade Exhibition 2011, July 11th to 13th, 2011, Convention Hall, Chulabhorn Research Institute, Bangkok, Thailand.