ISOLATION AND CHARACTERIZATION OF Tn5-INDUCED MUTANTS OF *Xanthomonas axonopodis* pv. *glycines* DEFICIENT IN PATHOGENICITY

INTRODUCTION

Bacterial pustule caused by *X. axonopodis* pv. *glycines* (Xag) is an important disease of soybean grown in tropical areas including Thailand (Prathuangwong, 1983, Prathuangwong *et at.,* 1990; Sinclair, 1982; Vauterin, *et al.,*1995). The favorable conditions for disease development are warm and highly humidity (Prathuangwong *et al.,* 1990 ; Prathuangwong *et al.,* 1996). The ability of plant pathogenic bacteria to cause disease in particular host plant depends on several factors including optimum condition of environmental aspects, plant physiology and development, and the expression of pathogenicity and virulence factors by the bacterial cells (Sigee, 1993). The interaction between plant and pathogen is a dynamic process that involves the exchange of signals. An important subset of pathogenicity genes performs regulatory functions that are likely required for the bacteria to adapt to the environmental and physiological changes encountered during infection. Plant-pathogen interaction including HR and disease induction are often affected by bacterial proteins that include harpin, avirulence protein (Leach and White, 1996) and pectate lyase (Kaewnum *et al.,* 2006). The expression of pathogenicity or pathogenicity-related genes is then, regulated by environmental factors, plant signals (carbon sources, organic nitrogen, and phosphate), and catabolite repression (Rahme *et al.,* 1992; Schulte and Bonus, 1992). The products of pathogenicity- or virulent related factors may function as signals molecule indicating that the environment is suitable for pathogen growth and initial pathogenesis (Sigee, 1993). Phytopathogenic bacteria utilize an apparent several strategies to access the nutritional haven afforded them in the intracellular spaces of plant tissues. They enter their hosts through natural openings or wounds on the plant surface (James and Collmer, 1996), where the environment is less harsh and rich in nutrients that include glucose, fructose, xylose, sucrose, and galactose (Padmanbhan *et al.,* 1974). Higher plants contain potentially a

lot of nutrients sources for many bacterial species in their environment. Most bacteria are small enough to pass through stomata and other natural openings into the apoplast as known. However, surprisingly few bacteria raid the nutrient stores of living plant cells, apparently because the metabolic involved in parasitism requires the function of specialists (James and Collmer 1996). In the terms of pathogenicity of bacterial pathogen, the ability to acquired nutrients from the host is essential for a pathogen to establish and infection. Bacteria occupy a wide variety of habitats and perform many ecological functions including nutrient or carbon utilization. Bacteria may use the gluconeogenesis pathway to synthesize glucose from non sugar C2 or C3 compounds or the intermediates of the tricarboxylic acid (TCA) cycle (Inui *et al.,* 1999 ; Osteras *et al.,* 1997 ; Osteras *et al.,* 1995). There are many reports have shown that gluconeogenesis is required for virulence of a number of animal pathogens such as *Salmonella enterica* serovar Typhimurium (Allen *et al.,* 2000), *Mycobacterium bovis* (Collins *et al.,* 2003), *M. tuberculosis* (McKinney *et al.,* 2000), and *Candida albicans* (Lorenz and Fink, 2001), but there is a few information involved in the pathogenicity of phytopathogenic bacteria especially in Xag.

Many bacterial plant pathogens produce extracellular products that contribute to their virulenc on host plant (Long and Staskawicz, 1993) with depend on protein secretion pathway (Pugsley, 1993). Expression of genetically determined pathogenicity and virulence factors is a key aspect in disease induction. Pathogenicity is the fundamental ability of a pathogen to cause disease, while virulence is the degree to which that pathogen affects the health of the plant (Sigee, 1993). Strain of Xag in Thailand and their population showed large differences in their virulence. Prathuangwong *et al.* (1996) analysed isolates of Xag using RAPD markers, and compared them with regard to plasmid content and pathogenicity. No relationship was detected between RAPDs and the geographical region from which the isolates were obtained, but there was a strong correlation between the RAPD pattern and pathogenicity. Prathuangwong and Ketsuwan (2003) classified 199 isolates of Xag collected from different regions in Thailand by rep-PCR and by pathotypic analyses on soybean. Several major rep-PCR products were correlated with highly (compared with weakly) aggressive isolates. The latter group had a high degree of similarity with

regard to rep-PCR profiles, whereas the highly aggressive isolate group was more heterogeneous. Moreover, the provided insight into those Xag were showed the diversity of pathogenicity on soybean and induction of the HR on several plant species Kaewnum *et al.,* 2005. Several factors which may contribute to the virulence of Xag have been reported, such as production of indoleacetic acid, cytokinin, extracellular polysaccharides, toxin, bacteriocins, cellulase and protopectinases (Hokawat and Rudolph, 1993 ; Fett and Dunn,1987). Cell-wall degradating enzymes secreted by microbial pathogens have been shown to be necessary for pathogenicity of most plant pathogenic bacteria (Salmond, 1994). However, the exact mechanism by which secreted cellulase effectors contributes to bacterial pathogenesis inside infected tissue is still a subject for further investigation. The understanding of genes involved in pathogenicity and virulent related factors of Xag and its process for recognition in host plants will likely lead to improve strategies for soybean bacterial pustule management.

OBJECTIVES

The objectives of this research are as follows.

1. Isolation of a pathogenicity mutant with Tn5, marker exchange technique and cloning of the gene that restored virulence proficiency of *X. axonopodis* pv. *glycines*.

2. Determining the secretory role affected by the restored gene.

3. To identify a pathogenicity mutant that is involved in defense-related signaling.

LITERATURE REVIEW

1. Importance of soybean in Thailand

Soybean (*Glycine max* L. Merr) is an important economic crop worldwide. The first record of soybean cultivation is from the north of china in the 11th century B.C. and spread into Japan, Korea and Southeast Asia between 200 B.C. and 300 A.D respectively (Hartman *et al.*, 1999). Utilitization of soybean is provides as an important protein source in the diets of many Asian nations and extremely valuable food and industrial products. Highly protein, nutritious, and minerals were contained in soybean seeds. Additionaly, soybean is an economic plant promoted to cultivate for consumption in the country and also export to foreign countries. Soybean has good potential for production in the tropics zone, but the yields are lower than those in temperate regions, because of poor nodulation by nitrogen-fixing bacteria and the lack of adapted cultivar.

Soybean has been planted since 2503 B.E in Thailan. The important spoybean growing area is mainly grown in the north and northeast regions of the country including Chiang Mai, Chiang Rai, Lum Pang, Nakhorn Sawan with three growing seasons for planting, during May to August, June to November, and December to April respectively. The recommended cultivars of soybean are SJ 4, CM60, KKU35 and Rachamongkol1 which are planted in different areas. However, soybean product in Thailand is not enough for both human consumption and use in animal feed materials. Total of soyben seed product show approximately 500,000 tons / year when the total demand of soybean seed materials is more than 600,000 tons. Diseases caused by several pathogens including fungi, bacteria and virus are mainly problem to soybean production (Prathuangwong, 1989, Prathuangwong *et al.,* 1996). Impotant diseases which significantly affected on both of quality and quantity of soybean produced such as rust, anthracnose, downy mildew, bacterial pustule, sudden death, and viral diseases which are negative effected on soybean production farm in Thailand. The disease distribution and severity observed were difference based on the different climate of soybean cultivation regions. (Prathuangwong *et al.,* 1996).

2. Soybean bacterial pustule, causal pathogen, and disease control

Bacterial disease of soybean occur worldwide and cause limited production during years of warm temperature and high moisture. The most common bacterial incited diseases are bacterial blight and bacterial pustule. The prevalence and severity of these diseases vary considerably from year to year which depend on the differences in weather patterns and cultivar of soybean. Bacterial pustule caused by *X. axonopodis* pv. *glycines* (Xag) (Parathuangwong, 1983; Sinclair, 1982; Vauterin, *et al.,*1995) is a worldwide bacterial disease of soybean which reveals more severe infection in the moderate to warmer areas. The disease occurs as important distributes in many soybean growing countries such as Argentina, Australia, Bolivia, Brazil, Cambodia, Canada, China, India, Japan, Malaysia, Nicaragua, Nigeria, Sudan, Taiwan, United states, (Sinclair and Dhingra, 1975) and Thailand (Prathuangwong *et. al.,* 1983). The diseases can be severe especially when the soybean is 30-40 days old (Prathuangwong, 1984) and affect both quality and quantity of soybean product. The outbreak of bacterial pustule was encountered in the late rainy season from July to September which it haved a favorable conditions of hot weather and periodic rain.

According to the investigation, the disease is widespread in soybean growing areas in the north, central and northeast regions of Thailand (Prathuangwong *et al.,* 1996). During 1976, the outbreak was quite severe since the recommended varieties called SJ 1, SJ 2, and SJ 4 were susceptible to the disease (Prathuangwong, 1983, Prathuangwong, 1984, and Prathuangwong *et al.,* 1996). The causal bacterium penetrates the soybean plant through natural opening and wounds (Sinclear, 1982) and multiplies intercellularly. The level of attack was associated with the number of stomata on the under surface of leaves. Several factors which may contribute to the virulence of Xag have been reported, such as production of indoleacetic acid and cytokinin (Fett and Dunn, 1987), extracellular polysaccharides toxin (Hokawat and Rudolph, 1993), bacteriocins (Fett *et al.,* 1987) or cellulase and protopectinases (Hokawat and Rudolph, 1993). Additionally, bacterium also secretes amylase, gelatinase and protease.

The disease symptoms primarily occured on the foliage is minute pale green spots with elevated center on either or both leaf surfaces. Thereafter, a small, raised, light- colored pustule forms in the center, usually in lesion on the under surface of the leaf, that some time may confuseed with the typical symptom of bacterial leaf blight caused by *Pseudomonas syringae* pv. *glycenea* (Sinclair, 1982). Moreover, the symptom of bacterial pustule has vary from minute specks to large, irregular, mottled brown areas that arise when smaller lesions coalesce. In later stages, dried, broken remnants of pustules may be seen on small brown necrotic areas surrounded by narrow yellowing haloes. Leaves then, become ragged when dead areas are torn away by the wind. Pustules are mainly formed by hypertrophy and also hyperplasia with surrounded by yellowing haloes (Prathuangwong, 1984; Sinclair, 1982). The symptoms may develop on stems and pods of susceptible varieties. The pustule pathogen commonly survives in soybean seeds and also in soybean crop debris depends on the environment, cultivars and virulence of the bacterial strain. More recently, there has been research and development of soybean cultivars for resistance to disease such as Sukhothai 1 (ST1), Sukhothai 2 (ST2), and Chiang Mai 60 (CM 60) (Prathuangwong and Amnuaykit, 1989). Symptoms on resistant soybean varieties have occurred as small chlorotic spots but no well-defined pustules or light green chlorosis or fewer and smaller pustules. The seriously damaged leaves causing defoliation in susceptible varieties resulted in 15-50% yield losses, giving small, light and low capacity soybeans. (Prathuangwong and Amnuaykit, 1989).

X. axonopodis pv. *glycines* (Xag) is a negative bacteria, rod shaped, does not form spores, has a single flagellum and a size of 0.2-0.9 x 0.57-2.59 µ. The optimum temperature for growth is 30-33°C. The unique characteristic of Xag are that it can produce catalase enzymes but not urease enzymes and is capable of digesting starch, gelatin, pectate and tween 80. This pathogen can produce hydrogen sulfide from peptone, cysteine and sodium thiosulfate to digest esculin and protein in milk and it is durable in 5% of salt medium. Xag can produce acid when tested with other sugars ; arabinose, xylose, glucose, manose, fructose, galactose, lactose, sucrose, maltose, threhalose, raffinosa, cellubinosa and glycerol. Characterization of colonies on media such as nutrient glucose agar, beef fusion agar and wakimoto’s agar found them to be

yellow, smooth rimmed, produce yellow pigment and dissolve in alcohol but not dissolve in water (Sinclear, 1982). Wakimoto's agar is situation growth at 30^0C, pH 6.8 (Hokawat and Rudolph, 1993), where as colonies on modificatio medium for Xag (MXG) is green convex shaped of smooth margin and fludial.

Our previously research have found that Xag strains exhibit variable groups as shown by DNA fingerprint (Prathuangwong *et al.*, 1996 ; Prathuangwong and Ketsuwan, 2003) that may complicate efforts to construct or select soybean plants resistant to all strains of Xag. The utility of a recently developed methods to classify bacteria on the basis of their genomic fingerprint patterns was investigated, using collections of both symbiotic and pathogenic plant-associated bacteria. The polymerase chain reaction (PCR) is the in vitro, primer-directed enzymatic amplification of nucleic acid. This technique has been used in many diverse applications including Xag. Random amplified polymorphic DNA (RAPD) analysis is DNA fragments amplified by the PCR using short (generally 10 bp) synthetic primers of random sequence. RAPD is a commonly used molecular marker in genetic diversity studies. RAPD have been used for many purposes, ranging from studies at the individual level (e.g. genetic identity) to studies involving closely related species. Due to their very high genomic abundance, RAPDs have been also applied in gene mapping studies.

Prathuangwong *et al.* (1996) analyzed the Xag genome which regard to plasmid and virulence diversities using RAPD. No relationship was detected between RAPD pattern and soybean growing region from which strain were obtained. There was a strong relationship between disease reaction and results of RAPD reaction type but less relation between disease reaction and plasmid profile. Both assays of symptom expression and RAPD were capable of distinguish strongly from weakly aggressive strains where the correlation between the presence of plasmid and the variability of pathogenicity remain to be determined.

Morever characterization genomic fingerprinting of bacteria is based on the use of DNA primers corresponding to naturally occurring repetitive elements in bacteria,

such as the REP, ERIC and BOX elements, and the PCR reaction (rep-PCR). Rep-PCR fingerprinting is a highly reproducible and a simple method to distinguish closely related strains, to deduce phylogenetic relationship between strains and to study their diversity in a variety of ecosystems. Rep-PCR genomic fingerprinting coupled to computer assisted phylogenetic analysis and library search programs will constitute a useful method for the identification or diagnosis of plant pathogenic, as well as symbiotic bacteria (de Bruijn, 1992; Louws *et al.,* 1994; Louws *et al.,* 1995). This technique has already been successfully used to study the population structure of plant pathogens and to follow greenhouse inoculation. It therefore is a useful molecular approach in molecular microbial ecology. Thus, the rep-PCR technique appears to be a rapid, simple, and reproducible method to identify and classify *Xanthomonas* and *Pseudomonas* strains, and it may be a useful diagnostic tool for these important plant pathogens.

Prathuangwong and Ketsuwan (2003) classified 199 isolates of Xag collected from different regions in Thailand by rep-PCR and by pathotypic analyses on soybean. Several major rep-PCR products distinguished weakly from highly isolates into two major genotype groups, Group 1 (weakly) and 2 (virulent). All group 1 isolates were relatively homogeneous with a high degree similarity. The majority of isolates belonged to group 2 and were genetically more heterogeneous. These results suggested that the BOX and ERIC-PCR method is useful for the identification of Xag Between the method was not useful for determine the geographic origin of the strains. They conclude that Xag collected from soybean are genetically heterogeneous and weakly and virulent isolates can be distinguished using rep-PCR.

Kaewnum *et al*. (2005) has reported that a 26 representative strains of Xag obtained from different soybean production areas of Thailand shown different with regard to aggressiveness on soybean and also differed in their ability to induce hypersensitive response (HR) on different non host cultivars. The results indicated that they have diversity on the strains of Xag and these information could be provided the basic knowledge for further studies on Xag-host plant interactions and genetically characterization of their pathogenicity.

Disease control of soybean bacterial pustule usually consists of using resistant cultivars, certified seeds, hot-water treatment of seeds followed by application of antibiotics or protectant fungicides, crop rotation, control of weeds and insects, and destruction of infected plants and debris in the field. In addition, the inappropriate use of antibacterial pesticide such as copper compound can have negative impact on health and environment, or may cause the emergence of resistance to the antibiotics in plant and human pathogens. Therefore, a reliable biological control agent may be a promising alternative for control of bacterial pustule disease in soybean (Prathuangwong

Kasem (2004) used two antagonistic bacterial strain, *Bacillus amyloliquefaciens* KPS46 and *Paenibacillus pabuli* SW01/4 which high efficiently inhibited growth and reduced infection of Xag by compatibility and secondary metabolites product.

Furthermore, various soybean production technologies such as specific planting, soil and nutrient management strategies, and the use of pathogen-free soil and seeds have resulted in a reduction in pests and diseases. Disease-resistant plants have also been introduced.

Rukayadi *et al.* (2000) reported that a nonpathogenic mutant of Xag strain M715 could reduce colonization of the soybean phyllosphere by virulent strain. Additionally they found that the epiphytic fitness was similar with the virulent wild type strain although the density of the mutant was slightly less than that of its parent. The mutant was able to survive for 16 days after inoculation on soybean leaves and maintained population densities of approximately 10^4 to 10^5 cells per g (fresh weight) of leaves.

3. Pathogenicity of plant pathogenic bacteria

Pathogenicity is the ability of pathogen to produce disease in a host organism. Microbial pathogen included plant pathogenic bacteria are express their pathogenicity by means of their virulence, a term which refers to the degree of pathogenicity of the pathogen. Hence the determinants of virulence of a pathogen are any of its genetic or biochemical or structural features that enable to produce disease. The relationship between a host and a pathogen is dynamic, since each modifies the activities and functions of the other. The outcome of an infection depends on the virulence of the pathogen and the relative degree of resistance or susceptibility of the host, due mainly to the effectiveness of the host defense mechanisms (Sigee, 1993).

In general, the entry of bacteria into the plant cell during the infection process leads to three types of interaction included hypersensitivity reaction (HR), disease reaction and no observable reaction (Lingrend, 1997). The interaction between bacterial pathogens and their plant hosts fall into two general categories. (1) Compatible, leading to intercellular bacterial growth and symptom development in the host, and (2) incompatible, resulting in the absence of observable diseases. Bacterial population in compatible interaction increases and distributes into other parts of the plant and induced disease symptom as systemic. On non-host plants, the incompatible interaction is often correlates with the elicitor of the HR when bacteria are introduced into leaf tissue and do not multiply. They can induce necrosis of cells at the infection site so the disease cannot spread to other parts of the plant (Wilis and Harbak, 1991). In additional, almost of plant pathogenic bacteria possess a conserved protein secretion system that is thought to transfer Avr (avirulence) proteins, with potential activities in both parasitism and defense elicitation, into plant cells. The *avr* genes may be acquired horizontally by these bacteria and compositions are highly variable. In the past year, heterologous expression experiments have revealed that the products of *avr* genes could be interchanged among different genera of bacteria with retention of secretion, pathogenicity, and avirulence activities, suggesting mechanisms for rapid coevolution of these parasites with changing plant hosts.

3.1 Hypersensitive Response and *hrp* Genes

The hypersensitive response (HR) of plants resistant to microbial pathogens involves a complex form of programmed cell death (PCD) that differs from developmental PCD in its consistent association with the induction of local and systemic defense responses. Hypersensitive cell death is commonly controlled by direct or indirect interactions between pathogen avirulence (avr) gene products and those of plant resistance (R) genes and it can be from the result of multiple signalling pathways (Lindgren, 1997). In this evidence, resistance genes of host plant could be detect the pathogen and change the membrane potential and ion permeability of the plasma membrane, resulting in suddenly cell death and formation of local lesions, which contain antimicrobial compounds. Next, the infected cells undergoing the HR will produce reactive oxygen species (ROS; oxidative burst), including super oxide anions, hydrogen peroxide, and hydroxyl radicals. Lipid peroxidation and lipid damage may be partially responsible for some of these cell changes and probably affect membrane function. Phenolics and phytoalexins, such as glyceollin (in soybean), and other compounds are synthesized cells surrounding the lesion. Callose, lignin, and HGRP are deposited, then pathogen related (PR) proteins, include 1,3-glucanase and chitinases are induced (Cheong *et al.,* 2000).

3.2 Pathogenicity and avirulence genes of Xanthomonas sp.

Avirulence (avr) genes are the most recent genetic variations in the evolutionary adaptation process between the host and pathogen. *avr* genes, first identified by Flor (1971) have been cloned from bacteria, fungi, and viral pathogens. The *avr* genes make a pathogen unable to induce disease on a specific variety of the host plant. In this way, *avr* genes determine the host range of the pathogen at the species and at the variety level. Their existence was predicted by the central tenet of the gene-for-gene hypothesis as the pathogen gene function required, in specific combination with either the direct or indirect product of the corresponding plant resistance gene, to trigger a plant resistance reaction (Leach and White, 1996).

The very existence of *avr* genes begs the question of why they would be maintained in bacterial populations, since they do, in fact, lower the fitness of strains carrying them on particular potential hosts. The simplest model explaining the existence of *avr* genes would be that they encode necessary components of the bacterial pathogenicity and/or virulence machinery, and that plant disease resistance genes have evolved to recognize these components (Leach and White, 1996). The following experimental regime is often used to test this hypothesis for essentially every new *avr* gene edentified. Marker exchange mutagenesis is used to disrupt the *avr* gene in the bacterial strain from which it was cloned. These marker exchanged strains are then tested on resistant and susceptible plant cultivars. The marker exchanged strain is expected to gain virulence on previously resistant hosts provided no other gene-for-gene interactions are operation in the particular strain-cultivar combination assayed. If the *avr* gene encodes a product absolutely necessary for pathogenicity, then the marker exchange mutant will lose pathogenicity on any previously susceptible host and will not gain pathogenicity on resistant hosts. This type of gene would be expected to be present in all strains of that particular bacterial pathovar. Unfortunately, this has been shown to be true only for the *avrBs2* gene from *X. campestris* pv. *vesicatoria* (Schulte and Bonas, 1992). A variant of this outcome is that the marker exchange mutant may only exhibit quantitatively lowered aggressiveness on some or all previously susceptible hosts, and will not gain virulence on previously resistant cultivars; the *avr* gene product is thus avirulence factor. This has been shown to be true for the *pthA* gene from *X.axonopodis* pv. *citri* (Swarup *et al.,* 1992). The highly related avrb6 gene from *X. campestris* pv. *malvacearum* and the *avr Rpm1* gene *from P. syringae* pv. *maculicola.*

The fact that most *avr* genes seem to have no role in pathogenicity or virulence suggests, among other possibilities, that either these genes in fact have no positive role in the pathogenic process, or that our infection assay systems bypass the stage in the normal plant-microbe interaction where these gene products are required. For example, one could imagine the *avr* gene products encode functions required at the switch point between epiphytic bacterial growth on the plant surface, and

intercellular growth as a pathogen. This stage of the interaction is often not assayed during hand or vacuum infiltration experiments.

Host-pathogen interaction can be thought of as a series of sequential events involving the pathogen and the host. The history of genetic studies of bacterial-plant pathogens has been comprehensively reviewed by Chatterjee and Vidaver (1986). Although *Agrobacterium, Erwinia* and *Pseudomonas* were increasingly studied during the 1970s while as *Xanthomonas* was a few reported (Chatterjee and Vidaver, 1986). Early research of transformation of *X. campesteris* pv. *phaseoli* and *X. oryzae* pv. *oryzae* have not been followed up and are difficult to evaluate. This demonstrated the feasibility of using gene cloning vectors derived from IncP Plasmid to transfer DNA into the bacteria. However, the convenience of using recombinant DNA techniques has meant that conventional genetic techniques have not been exploited further for the study of pathogenicity function.

However, within the genus *Xanthomonas*, several genes have been found associated with pathogenicity and virulence. These genes including the *avr* (avirulence), *rpf* (regulation of pathogenicity factors), and *hrp* (hypersensitive response and pathogenicity) genes are perhaps the most widely studied elements. The *avr* genes encode a known group of effector proteins responsible for controlling the ability of bacteria to elicit the hypersensitive reaction in resistant hosts (Leach and White, 1996) and may also participate in pathogenicity or virulence in compatible interactions (Ritter and Dangl, 1995 ; Swords *et al.,*1996). The *rpf* operon is thought to control the production of important pathogenicity factors, such as proteases, endoglucanases, polygalacturonate ligases, and extracellular polysaccharides (Barber *et al.,* 1997 ; Dow *et al.,* 2000). Finally, the *hrp* genes are thought to encode proteins involved in the type III secretion system, responsible for delivering effector proteins inside the host plant cells (Brodmann *et al.,* 2002 ; Hueck, 1998 ; Hyun-Ham, 1998). Even though pathogenicity and virulence of *Xanthomonas axonopodis* pv. *citri* have been traditionally associated with the activity of a single avirulence-like gene known as *pthA* (Swarup *et al.,*1991 ; Swarup *et al*., 1992) little is known about other gene products involved in these processes. In this respect, transcription profiling under

natural conditions may be a good alternative to identify all the elements involved in pathogenicity and virulence of this microbial pathogen. However, leaf spot pathogens, such as *X. axonopodis* pv.*citri* do not reach high population level in infected tissues and do not yield enough material (either bacterial cells or RNA) to conduct gene expression studies. Therefore, in an attempt to develop an alternative system for gene expression studies, an in vitro system was evaluated in order to determine whether it could be used to model pathogen responses to host tissues under controlled conditions. The two media selected were NB (nutrient broth), commonly used for growing this bacterium, and XVM2, suspected to mimic the environment of the plant intercellular spaces (Tung and Kuo, 1999 ; Wengelnik and Bonas, 1996).

4. Effect of environment and carbon source utilization involved in bacterial pathogenicity

Many environmental factors can be limiting to bacteria (Dommergues *et al.*, 1978) for their survival and pathogenicity. All of plant pathogenic bacteria survive comfortably in soil and ground moisture often are seed-borne, and the infection process can begin as the radicle emerges from the seed coat, signaling the beginning of vegetative plant growth. During this phase, bacteria from the abiota can access the emerging tissue and begin life as epiphytes on the plant surface. In addition, wounding of plant tissue, as a by product of rapid cellular expansion and division, allows resident bacteria ingress to intracellular domains of the host (Alfano and Collmer, 1996). If no wounds, or other natural openings such as hydathodes or stomata, are immediately available, the bacteria can multiply by several orders of magnitude as epiphytes under optimal conditions. In contrast, infection or pathogenic phase can occur later in the plant's growth cycle, as wounds and natural openings become colonized by epiphytically growing bacteria (Alfano and Collmer, 1996). This is the pathogenic phase of the plant-microbe interaction where the bacteria sense a change in their environment from plant surface to intracellular space. There is the reason to believe that global changes in bacterial metabolism occur at this cusp, and some of the genes sensing the environmental change have been identified (Rahm *et al.,* 1992). There are two genes, *hrp* and *avr* genes that could govern the critical early phase which

established pathogenesis. Other genes, encoding toxins and degradtive enzymes, become activated very shortly after, and the necrotrophic phase of the interaction begins in earnest. The classes of genes that determine the establishment of the pathogenic state encode pathogenesis factors, while genes encoding products governing the extent of colonization and the corresponding severity of disease, are termed virulence factors.

Therefore, however the pathogenicity of plant pathogenic bacteria can be expressed through several infection stages, for example invasion, recognition, multiplication of bacterial cell, production of virulence factors, and symptom development. These stages often occur in continuity and are difficult to recognize as in dependent phenomena. Among these stages, these are rather limited information available on bacterial multiplication in the compatible combination because this process has not been given much attention in host-parasitic interactions. The ability to acquire nutrients from the host is the one essential factor for a pathogen to establish the next an infection process. Phytopathogenic bacteria utilize an apparent cornucopia of strategies to access the nutritional haven afforded them in the intracellular spaces of plant tissues. Most phytopathogenic bacteria are nectrophic and thus have requirements to degrade for their metabolic use a certain amount of plant tissue (Dangl, 1995).

Invasion and multiplication of plant pathogenic bacteria through portals of entry such as natural opening and would is usually a passive phenomenon. Chemotaxis is sometimes referred to as the active response of bacteria to invasion. However, there is no conclusive evidence that chemotaxis is required for plant pathogenic bacteria to enter into host cell through the stomata or wounds. Furthermore, recently have many report about the communication among bacterial cell leaded to invasion process, terms as biofilm or quorum sensing. Bacterial quorum sensing depends on the production of acyl-homoserine lactone (AHL) signals and their detection by AHL receptors. When accumulated to an intracellular threshold concentration, AHL-receptor complexes bind regulated promoters and thus effect expression of the quorum sensing genes (Susanne *et al.,* 2003). Quorum sensing allows bacterial populations to behave as multicellular

organisms and mount a coordinated attack on their plant or animal hosts. Quorum sensing also plays important roles in plant-bacterial interaction, bacterial cells sense their population density through a sophisticated cell-cell communication system and trigger expression of particular genes when the density reaches a threshold. This type of gene regulation, which controls diverse biological functions including virulence or pathogencicity of plant pathogenic bacteria (Susanne *et al*., 2003). The previous report have shown that regulating the production of extracellular enzymes and EPS themselves are under quorum sensing control or are strictly regulated by the environment at the site of colonization (Poplawsky *et. al.,* 1998). However, *ppsA* in this study remains to determine whether it links to *rpf* encoded DS or DSF function of Quorum sensing in Xag. Additionally, the EPS and EPS-encoding genes of genus *Xanthomonas* have been well characterized (Harding *et al.,* 1987 ; Reinhard *et al.*, 1992 ; Chou *et al.,* 1997).

5. Glycolysis Pathway and Pathogenicity of Plant Pathogenic Bacteria

Glycolysis or gluconeogenesis pathway is the sequence of reactions that converts glucose into pyruvate with the concomitant production of a relatively small amount of ATP. Glycolysis can be carried out anerobically (in the absence of oxygen) and is thus an especially important pathway for organisms that can ferment sugars. For example, glycolysis is the pathway utilized by yeast to produce the alcohol found in beer. Glycolysis also serves as a source of raw materials for the synthesis of other compounds and energy. For example, 3 phosphoglycerate can be converted into serine, while pyruvate can be aerobically degraded by the Krebs or tricarboxylic acid (TCA) cycle to produce much larger amounts of ATP G6P in the cells.

In the terms of pathogenicity of bacterial pathogen, bacteria occupy a wide variety of habitats and perform many ecological functions including nutrient (carbon) utilization. Generally, the ability to acquired nutrients from the host is essential for a pathogen to establish and infection. In this case nutrient or carbon source is the one of basic elements. Bacteria may use the gluconeogenesis pathway to synthesize glucose from nonsugar C2 or C3 compounds or the intermediates of the TCA cycle when there

is not sufficient hexose in their niches (Osteras *et al.,* 1997). In additionally, plant pathogenic bacteria enters its host through natural openings (such as stomata) or wounds on the plant surface (Graham *et al.,* 1992), where the environment is less harsh and rich in nutrients that include glucose, fructose, xylose, sucrose, and galactose (Padmanbhan *et al.,* 1974). The interaction between plant and pathogen is a dynamic process that involves the exchange of signals. An important subset of pathogenicity genes performs regulatory functions that are likely required for the bacteria to adapt to the environmental and physiological changes encountered during infection. The expression of pathogenicity or pathogenicity-related genes is regulated by environmental factors (such as pH and osmotic strength), plant signals (such as carbon sources, organic nitrogen, and phosphate), and catabolite repression (Rahm *et al.,* 1992)

MATERIALS AND METHODS

1. Bacterial Strains, Plasmids, Medias, and Culture Condition

The bacterial strains and plasmids used in this study are listed in Table 1 and 2. Strains of *X. axonopodis* pv. *glycines* (Xag) were grown on nutrient agar (NA) or nutrient yeast extract (NY) agar (White and Gonzalez, 1995) at 28^{o}C. *Escherichia coli* strains were grown on Luria Bertani (LB) agar or broth and incubated at 37^{o}C. Most Xag strains were revived from stock cultures collected of department of plant pathology laboratory, Kasetsart University (Ketsuwan, 2003) with streak plate method (Schaad, 1988). Some strains were isolated in this study by cut the infected soybean leaves into 1-2 cm^{2}. The tissue was washed with surface sterilized for 3 min in a 10% dilution of household bleach (6% active sodium hypochlorite) and sterile distilled water (SDW) for 5 min. After rinsing in SDW, the tissue was grind in a droplet of SDW in a Petri dish and streaked onto agar NGA medium with a wire loop and incubated at 28^{o}C for 24-48 h. A yellow-pigmented bacteria single colony was purification by streaking onto NGA for Koch's postulation test on SJ 4 susceptible soybean cultivar. The cultures stored in 20% glycerol solution were kept at –20^{o}C and the routine use of slant cultures was kept at 4^{o}C. Bacteria from these slant cultures were streaked onto agar plates to obtain single colonies that were subsequently used for inoculation experiments. The optical densities of bacterial suspensions were determined with a spectrophotometer and the population of cell at various concentrations were counted by dilution plating. The population density of bacterial strains used in this experiment was varied from of 10^{4} to 10^{11} colony forming unit (cfu/ml). Media were amended with appropriate antibiotics at the following concentrations: ampicillin 100 µg/ml, kanamycin 50 µg/ml and tetracyclin 25 µg/ml for selection Xag mutant strains, *E. coli* carrying plasmid vector, and complemented strain respectively. Methods of plasmid introduction into Xag recipients of Xag mutant, gene extraction and expression, media, and culture condition involved were separately described in the following experiments.

Table 1 Bacterial strains and plasmids used in this experiment

Strain or plasmid	Relevant characteristics [1/]	Reference source [2/]
Escherichia coli DH 5α	F^- ϕ 80d*lacZ* △M △15 (*lacZYA-argF*) *U169 endA1 deoR recA hsdR17* ($r_k^- m_k^+$) *phoA supE44* λ- *thi-l gyrA96 relAl*	Invitrogen
X. axonopodis pv. *glycines* strain No.12-2	Laboratory wild type, spontaneous rifampicin resistance	KU / This study
X. axonopodis pv. *glycines* mutant strain KUMNTP2	Pathogenicity deficient::Tn5-Km^r	KU / This study
Plasmids		
pJB4JI	mob,$^+$ Mu,Tn5	Shizuoka University
pUC18	Amp^r cloning vector	Takara company
pRK2013	Helper vector : Km^r	Shizuoka University
pLAFR3	Derivative of pLAFR1, 23 kb, polylinker from pUC8, $lacZa^+$, Tc^r	Shizuoka University
pBluscript II S/K(-)	High copy number cloning vector; Amp^r	Stratagene

[1/] Amp^r = ampicillin resistance; Km^r = kanamycin resistance, Tc^r = tetracycline resistance, Gm^r=gentamycin resistance, Cm^r= Chloramphinical resistance.

[2/] KU = Kasetsart University.

Table 2 *Xanthomonas axonopodis* pv. *glycines* (Xag) strains use in this experiment

Xag Strain	Code strain	Source	Reference source[1/]
KU-K-46012	No.12-2	Nakhon Ratchasrima	This study
KU-K-44065	NKR13	Nakhon Ratchasrima	Stock culture, KU
KU-K-44069	NKR17	Nakhon Ratchasrima	Stock culture, KU
KU-K-44072	NKR21	Nakhon Ratchasrima	Stock culture, KU
KU-K-44089	BK01	Bangkok	Stock culture, KU
KU-K-44102	NO.15	Nakhon Sawan	Stock culture, KU
KU-K-44105	NO.21	Chaing Mai	Stock culture, KU
KU-K-45116	RE07	Khon Khan	Stock culture, KU
KU-K-45117	RE08	Leoi	Stock culture, KU
KU-K-45147	CM60-1	Nakhon Ratchasrima	Stock culture, KU

[1/] KU = Kasetsart University.

2. Pathogenicity Test

The different soybean cultivars including SJ4, SJ5, CM60, and ST1 were used in this study. All of soybean plants were grown individually in plastic pot containing commercial soil and maintain in greenhouse for 30 days or until used in the experiment. Watering and fertilizer were applied as recommended practical. The pathogenicity of Xag strains were tested by foliar spray inoculation. Bacterial cell suspension prepared by culturing strains at room temperature (28-30°C) for 48 h in NGA. Bacterial suspensions at OD_{600}= 0.2, which corresponded to a cell density of about 10^8 cfu/ml supplemented with 0.25 ml/l of adjuvant and Tension-T7 were sprayed onto foliage of the 25- day old of each different soybean cultivars. These cultivars were different resistant levels to bacterial pustule (Prathuangwong and Amnuaykit, 1989). Sterile water as a control was included in each trial. The inoculated plants were observed at 4-14 days after pathogen inoculation and scored for the severity of pathogenicity as method described by Prathuangwong *et al.* (1993). Leaf area affected was evaluated by placing the stencil card of 4 x 7 cm with 9 circles of 0.5 cm in diameter punched out on the infected leaves (Fig.1). The number of holes with lesions and the total number of hole on leaf (9 holes) were calculated for percentage of infection. The representative strains which showed high disease severity will be used for studies in the next step.

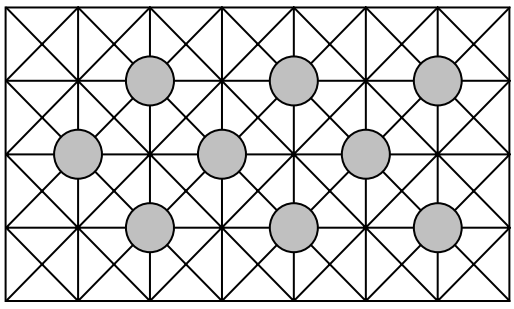

Figure 1 The stencil card of 4x7 cm with 9 circles of 0.5 cm in diameter punched out used for measuring soybean bacterial pustule severity described by Prathuangwong *et al.* (1993).

3. Hypersensitive Response Characterization

All of Xag strains in Table 3 were tested for their ability to induce hypersensitive response (HR) on various nonhost plants includind tobacco (*Nicotiana tabacum* cv. Xanthi,) pepper (*Capsicum annuum* cv. Cayenne), and tomato (*Lycopersicon esculentum* cv. Seedatip-II). All of nonhost plants were grown from seeds in plastic pot containing commercial soil and maintained under greenhouse condition. Xag suspension cultured in NB medium were pelleted down and resuspened in water. Bacterial suspensions at A_{600nm}= 0.5 OD, which corresponded to a cell density of $5x10^9$ cfu/ml were infiltrated into the leaf mesophyll of tobacco (4 to 5 weeks old), tomato (20 to 25 days old), and pepper (15 to 20 days old) using a 1-ml hypodermic syringe without a needle (Wei *et al*., 1992). Infiltrated zones were observed for development of tissue collapse within 24-48 h post infiltration. The experiment was repeated twice. The effect of cell density of each Xag strains on HR induction was also determined using serial suspension at optical density (OD at $A_{600\ nm}$) of 0.2, 0.5, 1.0 and 1.5 accorded to cell density of $2x10^8$, $5x10^9$, $5x10^{10}$, $5x10^{11}$ cfu/ml respectively as methods described by Kaewnum (2005). The Xag mutant strains used in the study were selected base on their virulence on host and HR on each nonhost plant.

4. Transposon Mutagenesis

4.1 Bacterial and plasmids preparation

The representative virulence strains (wildtype) of *X. axonopodis* pv. *glycines* was selected and grown at room temperature (28-30^{o}C) on NA plate or yeast extract peptone agar (YP). *E. coli* strain S17-1 which contain the donor plasmid pSUP2021, strain SM-10 which contain the donor plasmid pJB4JI and strain HB101 which contain the helper plasmid pRK2013 were grown routinely with LB agar or LB broth (Maniatis, 1982) supplemented with an appropriate concentration of kanamycin (50 µg/ml) and incubated at 37^{o}C.

4.2 Triparental mating

The suicide plasmid carrying the transposon Tn5 fragment, pJB4JI and pSUP2021 were separet introduced into Xag strain No.12-2 chromosome by triparental mating. The donor (*E. coli* with pJB4JI) and helper (*E. coli* HB101 with pRK2013) strains cultured in LB containing kanamycin and incubated overnight at 37^0C at logarithm phase whereas Xag was separate cultured in LB broth for 18-24 h. Bacterial cultures at their optimum growth rate was monitoring with the optical density of 0.5 OD (A_{600nm}) with spectrophotometer (CECIL CE1011). Each bacterial suspenrion were mixed in the 1.5 ml eppendrof with a ratio 1 : 1 : 1 of recipient : donor : helper respectively. Mixed cell suspension was spreaded on yeast-extracted peptone agar (YPA) supplemented with kanamycin and incubated at room temperature for 1-3 days or until the yellow colonies were occured. Each bacterial patch was scraped and resuspended in 1 ml of sterilized water and diluted for appropriate cell density at 10^{-3} of serial dilution. The 50 µl of cell suspensions were plated and spread onto the same media and incubated at room. Yellow colonies grown on tested plate were recorded and restreaked several times onto NA plates supplemented with kanamycin a before tests for pathogenicity and for hypersensitive response (HR).

5. Screening of Nonpathogenic Mutant Strains

5.1 Characterization of disease induction

The defective pathogenicity of Xag mutant strains were determined by inoculating the mutant strain with cotyledon and *in planta* bioassay onto susceptible soybean cultivar SJ4 comparing with wildtype strain.

5.1.1 Cotyledon bioassay

Inoculation of detached soybean cotyledons was done as method described by Hwang *et al.,* (1992). The 10-day old soybean seedlings cultivar SJ4 grown in a greenhouse were surface sterilized with 0.5 % sodium hypochloride for 3

min and washed with sterile distilled water for 5 min. The center of each cotyledon was punctured with multiple pins. On the wound site, 20 µl (10^8 cfu/ml) of each mutant suspension and wildtype strains of Xag were dropped. Inoculated cotyledons were kept in high moisture conditions with 16-h photo period at 30^{o}C. The non-pathogenicity mutants were observed and identified by absence of chlorotic symptoms around the inoculation site within 3 to 4 days after inoculation compared with positive and negative controls using the wild type strain of Xag and distilled sterile water inoculations, respectively.

5.1.2 Pathogenicity test on soybean

The 20 ml of each Xag mutant suspension at 10^8 cfu/ml was foliar spray inoculated on the 25 to 30 days old susceptible soybean cultivars (SJ4) as method of Prathuangwong (1984). The inoculated plants were incubated under greenhouse condition with recommend practical cultivation. The pathogenicity of each mutant on inoculated soybean plants was performed and observed at 7 to 14 days after inoculation. The wildtype strain and potassium phosphate buffer (10 mM, pH 7) or steriled dislitted water (SDW) used as a positive and negative control respectively were included in each trial.

5.2 HR induction

The ability on HR induction of Xag mutant and wildtype strains were preliminary determined on resistance soybean cultivar ST1 and nonhost plant, tomato and tobacco as method described below. All of tested plants, soybean, tobacco, and tomato used in this experiment were individual grown from seeds in plastic pot containing with the commercial soil. All of tested plants were maintain in greenhouse condition until used in the experiment. For bacterial suspension, the cell cultures of both Xag mutant and wildtype strains cultured in NB medium were pelleted down and resuspened in sterile distilled water. Bacterial suspensions were adjusted at optimum concentration as methods described. Bacterial suspensions at 0.2 OD (A_{600nm}) which corresponded to a cell density of about $2x10^8$/ml was infiltrated into the leaf

mesophyll of each tested plants including 14 days olds of soybean ST1 cultivar, 4 to 5 weeks old of tobacco, and 3 or 4 weaks old of tomato respectively by using a 1-ml hypodermic syringe without a needle (Wei *et al*., 1992). Infiltrated zones were observed for development of tissue collapse and necrosis for 24-48 h post infiltration. The resulting reactions were given symbolic scales from + to ++++ ; - = no visible reaction; + = slight necrotic spots visible in infiltrated area; ++ = clearly necrotic spots visible in infiltrated area, +++ = tissue collapse and necrosis at margins of infiltrated region; and ++++ = complete collapse and necrosis of the entire infiltrated area.

To analyze bacterial growth *in planta*, suspension of 0.2 OD (A_{600}) in sterile distilled water was infiltrated into leaves of resistance soybean cultivar (ST1). Leaf discs of 5 mm diameter of soybean were cut from the center of the infiltrated zone at the intervals from 0, 6, 12, 24, 48, 72, 96 and 120 h post infiltration (four discs for each treatment and each time interval were investigated). Samples were placed individually in 1 ml SDW, triturated and dilutions at 10^{-7} to 10^{-12} and plated on LB agar medium supplemented with kanamycin (50 µg/ml) and refampicin (25 µg/ml) for detected Xag mutant and wildtype strains respectively. The population density of bacterial strains counted from each tesed plated were calculated and adjusted by colony forming unit per ml per leaf disc (cfu/ml).

6. DNA Manipulation and Hybridization Analysis

Total genomic DNA of nonpathogenic mutants was isolated by chloroform extraction and ethanol precipitation essentially as method described by Sambrook *et al*. (2001). Cell culture of bacteria (1.5 ml) at 18-24 h was pelleted by centrifugation at 8,000 rpm for 1 min. The pellet was resuspended in lysis buffer (40 mM Tris-acetate pH7.8, 20 mM Sodium acetate, 1 mM EDTA, 1% SDS) and 5 M NaCl, pelleted again by centrifugation as described in previous step, kept the supernatant, RaseA was added and incubated for 1 h at 37°C. DNAs were extracted with chloroform. Samples were centrifuged at 12,000 rpm for 5 min followed by adding chloroform-isoamylalcohol (24:1) and centrifuging as described above. The upper phase (only DNA) was transfered to a clean eppendorf tube and precipitated by adding isopropanol (V/V), and

centrifuged at 12,000 rpm for 15 min. The DNA was placed in a microcentrifuge tube within 100 µl of 70% of ethanol and centrifuged 12,000 g for 15 min. Ethanol was decanted and the pellet was dried in a vacuum. The DNA was dissolved in 30 µl of TE buffer. The quality of the chromosomal DNA was determined by agarose gel electrophoresis on a horizontal, 0.8 % agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 100 volts constant for 90 min using 1X TBE as a running buffer. The gel was stained with 0.5 µg of ethidium bromide per ml for 10 min and briefly then destined in water before visualizing under UV light and photographed over a transilluminator (GDS 800, Complete Gel Documentation Analysis System) and kept at 4 ^{o}C. The transposon inserted fragment in mutant chromosome was detected by PCR and southern hybridization as method describe below (Sambrook *et al.,* 2001).

6.1 PCR analysis

Primers using for DNA detection were designed from the nucleotide sequence of the transposon fragment including primer Tn5 Forward ; 5' TGA GCT GTA ACA GCC TGA CCG C3' and Tn5 Reward ; 5' CAC CAC ATT CCG CAC CGT AG 3'. DNA was amplified in a total volume of 50 µl. The reaction mixture contained 5 µl of 10 X buffer (500 mM KCl, 100 mM Tris.Cl ; pH 9 at 25^{o}C), 1.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate (Boehringer Mannheim), 25 pmol of each primer, and 2.5 U of Taq polymerase (Qiagen). The amount of template DNA added was 100 ng of purified total bacterial DNA. A total of 30 amplification cycles were performed in an automated thermocycler (P-100). Each cycle consisted of 5 min of pre-denature at 95°C, 30 sec of denaturation at 95°C, 30 sec of annealing at 58°C and 30 sec of extension at 72°C and the last extension step was extended to 7 min. Amplified DNA was detected by electrophoresis with 0.8% agarose gels in 0.5X TBE buffer (44.5 mM Tris base,4.5 mM Boric acid,1 mM EDTA), stained with 0.5 g of ethidium bromide per ml, and then photographed over a UV transilluminator (GDS 8000; Complete Gel Documentation Analysis System).

6.2 Southern hybridization analysis

For probe preparation, the appropriate DNA fragments from donor *E. coli* strain SM-10 which contained transposon (Tn5) and kanamycin resistance genes fragment was purified with Plasmid Extraction Kit (Bioneer). Tn5 region was amplified by Tn5-specific primer and optimum PCR reaction as method described above. PCR product was separated by 0.8 % agarose gel-electrophoresis. The DNA band of the appropriate size of Tn5 extracted by Gel Purification Kit (Bioneer) was labeled with non-radioactive DIG-High Prime (Bochringer Mannheim) as recommend by the suppliers for using on the next process.

Southern hybridization analysis was performed by transfer DNA from the 0.8% agarose gel onto nitrocellulose membrane using a method described by Sambrook *et al*., (2001). The genomic DNA of mutant strains of Xag was digested separately with endorestriction enzyme *EcoR*I. Appropriate amounts of each digested DNA were transferred to fresh eppendorf and 0.5 volumes of 6X sucrose gel-loading buffer was added then, the fragments of DNA was separated by gel electrophoresis. The gel was stained with etidium bromide and the DNA bands were visual observed under UV transluminator.

After fractioning the DNA by gel electrophoresis, transferred to a glass baking dish and the DNA denatured by soaking in a denaturing solution. Denatured DNA were transferred from the agarose gel onto a nitrocellulose membrane, Fixed DNA to the membrane and probe hybidization with DIG-labled Tn5 region probe performed using the method described by Sambrook *et al.*, (2001). The filter membrane of hybridized DNA was detected at room temperature by incubating for 30 min in antibody-conjugate solution followed by dark incubation in nitroblu tetrazolium salt and phosphate solution (overnight). The reaction was stopped by soaking the nitrocellulose membrane in buffer4 (10 mM Tris-HCL, 1 mM EDTA pH 8.0) for 5-10 min and dried at room temperature before detection of hybridization signals.

7. Cloning of Transposon Flanking Sequences

Chromosomal DNA of nonpathogenic mutant strain containing Tn5 fragment was digested with *EcoR*I and cloned into plasmid vector pUC118 prior to sequencing by heat shock technique. Tn5 specific primers used for confirmed target clone by PCR as methods described above. The entire DNA fragments of mutant strain were amplified by PCR analysis. The predicted length of the amplified PCR DNA in target clone was subcloned into pGEM–T easy (Promega, Madison, WI) and sequencing. When the large fragment genes of target clone were undertaken, it was separated by restriction enzyme *Sac*I and subcloned in to pBluescript S/K (+). The ligation mixture was added with 6 µl of *Sac*I fragment, 3 µl of 10 x T4 DNA ligase buffer, 2 µl of T4 DNA ligase (Bio-lab), 3 µl of vector plasmid and 6 µl of dH_2O. The reaction was immediately mixed and incubated at 16^{o}C for 12 h. A 10 µl of the ligated products was added to 50 µl of competent *E. coli* DH5α and DH10B cell (Stratgene) by heat shock method. Transformants were selected on LB agar containing 50 µg/ml kanamycin and ampicillin. Kanamycin and ampicillin resistant recombinants were picked within 16-24 h after incubation at 37°C and grown in LB broth containing 50 µg/ml kanamycin and ampicillin at 37°C for 16 h with vigorous shaking. Plasmid DNA from transformants with the appropriate phenotype, contained Tn5 flanking fragment was isolated by the alkaline lysis miniprep procedure (Sambrook *et al*., 2001). Restriction analysis and PCR analysis detected the pBluescript S/K (+) plasmid presumably containing Tn5 flanking DNA. DNA sequencing was done at the Genetic Unit Center Shizuoka University with an Automated DNA Sequencer, and at the Bioservice Unit, National Science and Technology Development Agency (NSTDA), Thailand. For DNA and protein sequence analysis was done with the BLAST program with the National Center for Biotechnology Institute (NCBI) and DNASTAR (Madison, WI.) software packages.

8. Complementation Assay

To complementation pathogenicity minus strain, the target gene was subclone from Xag wild type strain No.12-2 and introduced into mutant strain KUMNTP2 as

the process described by Sambrook *et al.* (2001). A 2,500 bp fragment containing the pathogenicity related gene was amplified from wild type by PCR using the forward primer 5'ACTGGATCCTTTCAGCGGTGATACCGGTTCC and reverse primer 5'CCCAAGCTTGGCGGCCGCTCCCGCAGCCGCC. The amplified pathogenicity related gene initially was cloned into the pGEMT-easy and subcloned into a unique *Eco*RI site in pBluescript S/K (+). The construct was digested with restriction enzyme *Bam*HI and subcloned into low copy vector, pLARF3 and transform into competent cell *E.coli* S17. The complementation *E. coli* strain were screen on selective medium, LB supplemented with 25 tetracyclin 25 μg/ml. A complemented plasmid was isolated and mobilized into Xag mutant strain by triparental mating and selected the complemented colonies by LB medium supplemented with ampicillin and tetracycline at 100 μg/ml and 25 μg/ml respectively, and confirm as transformants by plasmid isolation and PCR analysis using primer described above. The complemented strain was determined the pathogenicity and gene functions on host plant, HR on tobacco and others experiment as methods described below comparing with mutant and wildtype strains.

9. Characterization of Nonpathogenic Mutant and Pathogenicity Related Function Analysis

9.1 Biochemical characterization

Criteria for identifying the strains included gram stain, oxidase test, gelatin hydrolysis, starch hydrolysis, leaven formation, lipolytic activity, salt tolerance, carbon utilization, and growth on MXG medium (Khundet, 1989) were investigated as method described by Schaad (1988).

9.1.1 Gram reaction with 3% KOH test

The simple KOH technique can be used as a rapid test for presumptive identification between Gram-positive and Gram-negative bacteria. A loopful of growth from a colony of each Xag wild type, mutant strain and

complemented strains was emulsified on the surface of a glass slide in a suspension of 3% KOH. The suspension was stirred continuously for 60 seconds then gently pulled the suspension with loop. The test was considered positive reaction if stringing occurred within the first 30 seconds of mixing the bacteria in KOH solution and this sample strain.

9.1.2 Oxidase test

Cultured each Xag wildtype, mutant strain and complemented strains on NA plate for 24 h at room temperature. Rub a small loopful of colonies of each strain on a filter paper impregnated with 1 % (w/v) aqueous tetra-methyl-p-phenylenediamine dihydrochloride solution (freshly made). The strain tested was recorded with oxidase-positive A toothpick is recommended since traces of iron can catalyze the oxidation of the phenylenediamine compound.

9.1.3 Gelatin hydrolysis

Colonies of Xag wildtype, mutant strain and complemented strains cultured on NGA for 24 h was stabbed with a loop of inoculum onto the center of the gelatin medium tube.The teated medium tubes were stored at room temperature (25^0C) for 48 h, then the inoculated tube was observed for gelatin hydrolysis by chilled at the 4^0C for 30 min or until the control treatment indicate areas of gelatin hydrolysis.

9.1.4 Starch hydrolysis

Each bacterial cuture was streaked onto nutrient starch agar. The inoculated plate was incubated for 5 days. Flood plates with Lugol’s iodine solution were checked. Clear and colorless zones indicate starch hydrolysis being positive.

9.1.5 Leven formation

Xag wildtype, mutant strain and complemented strains were individually streaked onto NGA to which 5% (w/v) sucrose has been added. White mucoid, dome-shaped colonies after 3 to 5 days incubation indicate a positive reaction.

9.1.6 Lipolytic activity (Tween 80)

A colony of each Xag wildtype, mutant strain and complemented strains were streaked onto NGA for 24 h at 28 ^{0}C and restreaked onto Tween medium. After 7 days, growth was determined the yellow pigment with white crystal in the bacterium.

9.1.7 Salt tolerance

Xag wildtype, mutant strain and complemented strains were cultured separately on NGA for 24 h. The bacterial culture was transferred with a loopful into the 5 %, 7 % of NaCl solution in NB medium, shaking at 225 rpm for 48 h at room temperature (25^{0}C).

9.1.8 Carbon utilization

This should be observed from agar slants of medium-C of Dye (Schaad and Russell, 2001) by adding carbon source utilization (glucose, sucrose, dextrose, manital, maltose and fructose), 0.5 % (v/v) aseptically from filter-sterilized stock solutions. A culture was examined for carbon utilization after 7 days of inoculation. A yellow color was indicated positive of carbon utilization.

9.1.9 Growth on MXG medium

X. axonopodis pv. *glycine* wildtype, mutant strain and complement strains were cultured on NGA for 24 h and cross streaked onto MXG medium for 72 h.

The colonies with green convex shaped of smooth margin and fludial character revealed Xag type strain.

9.2 Characterization of pathogenicity related factors

9.2.1 Bacterial growth *in vitro*

To analyze bacterial growth *in vitro*, a single colony of Xag wildtype and mutant including complement strains were streaked on LB plates and incubated at 28°C for 48 h for wildtype and mutant strains and 37^0C for complement strain. Then, one loopful of bacterial cells was suspended in LB broth incubated at optimum temperature as methods described above on incubator shaker for 16-18 h at 200 rpm. Cell suspension of each bacterial strain was adjusted by sterilized distilled water. The optical densities of bacterial suspensions at 1.0 OD at 600 nm absorbance wave length were determined with a spectrophotometer. The 1.5 ml of each bacterial suspension was separatly added into the 200 ml of minimal medium (M9) supplemented with 0.5 % glucose and M9 supplemented with 0.25 % sodium acetate compared with cultured in M9 alone. All of tested media were incubated at room temperature on incubator shaker at 200 rpm. The population density of each bacterial strain and in each tested medium were measured at 0, 3, 6, 9, 12, 18, 24, 36, 48, 60, 72, 84, 96, and 120 h after incubation with spectrophotometer.

9.2.2 Bacterial growth *in planta*

To verify whether the reduced virulence was correlated to the reduced growth rate of the pathogen, the investigation on the growth rate of bacteria *in planta* was done. The 30-day old of susceptible soybean cultivar (SJ4) grown in the plastic pod contained with commercial soil and maintained in greenhouse condition was tested using tissue infiltration methods. Bacterial cells of each Xag wildtype, mutant, and complement strains grown in LB medium at 28°C with shaking at 200 rpm for 18-24 h were prepared the cell suspensions at a concentration of $1x10^8$ cfu/ml (0.2 OD (A_{600nm}) with sterile distilled water. Ten µl of each bacterial cell suspension

was inoculated by the infiltration assay into mesophyll of soybean leaves using a 1-ml hypodermic syringe without a needle. The tested soybean plants were placed and maintained in green house condition all of incubation period. Leaf discs of 5 mm diameter tissue samples were cut from the center of the infiltrated zone at the intervals from 0, 6, 12, 24, 36, 48, 72, 96, and 120 h post infiltration (four discs for each treatment and each time interval were investigated). Samples were macerated and placed individually in 1 ml of sterile distilled water, triturated, and isolated by plating for individual colonies on YP medium containing the appropriate antibiotics including 50 ug/ml rifampicin, 50 ug/ml kanamycin, and 50 ug/ml of tetracycline for wild type, mutant, and complementation strains respectively. For all experiments at least three leaf panels per three plants for each treatment were infiltrated and the experiment was repeated several times. The development of disease symptom or HR-like symptom was detected at interval of 48, 72, 96, and 120 h post infiltration after infiltration.

9.3 Effect of bacterial density on disease induction

Wildtype and mutant strains of Xag were tested for the capability to induce disease symptom on 30 days old susceptible soybean cultivar (SJ4) with tissue infiltration and foliar spray inoculation comparing with the complemented strain. Each bacterial cultured in NB at 24 h was prepared for cell suspension with sterilize distilled water. Cell suspension of each strain was adjusted by varying the concentration of inoculums source at 0.2, 0.5, and 1.0 OD (A_{600}) which corresponded to a cell density about 10^8, 10^9, and 10^{11} cfu/ml respectively. The 20 ml of each bacterial suspension at 0.2 OD was foliar spray on soybean plant and kept in greenhouse condition. The pathogenicity of each strain on inoculated soybean plants was performed and observed at 5, 7, and 14 days after inoculation. The wild type strain and potassium phosphate buffer (10 mM, pH 7) used as a positive and negative control were included in each trial respectively. For tissue infiltration assay, 10 µl of each bacterial cell suspension at concentration of 0.2, 0.5 and 1.0 OD ware individually infiltrated into mysophyll of 10 days soybean leaves. All of infiltrated plants were kept and maintain in greenhouse condition. Initial symptom as necrotic lesion was observed on infiltrated leaves at 0, 12, 24 36, 48, 72, and 96 h over through 14 days post infiltration.

9.4 Effect of carbon source utilization on pathogenicity

In planta experiment, carbon utilization of nonpathogenic mutant strain and pathogenic including complemented strains on host plant were investigated. All strains were cultured in 200 ml LB and incubated on rotary shaker 150 rpm at room temperature for 24 h. Cell suspension of each bacterial strain was adjusted by sterilized distilled water and the optical densities of bacterial suspensions at 0.2 OD (A_{600nm}) were determined with a spectrophotometer. In each bacterial strain, suspension was supplemented with 10% (V/V) glucose before inoculation onto soybean plant with infiltration and foliar spray as method described above. The population density of each bacterial strain was evaluated at interval of 0, 6, 12, 24, 48, 72, and 96 h after inoculation with dilution plate count method. The development of disease symptom or HR-like symptom was detected at 0, 12, 24, 48, 72 and 96 h after infiltration. For the foliar spray inoculation, each 20 ml of bacterial suspension was sprayed on soybean leaves. The frequency of spray inoculation varied from 1, 2 and 3 time at 24 and 48 h after the first inoculation. After inoculation, plants were maintained in the greenhouse until symptom was assessed every day from 4 to14 days after inoculation with the method described by Prathuangwong *et al.* (1988).

9.5 Effect of bacterial cell density and glucose on HR induction

For the ability of HR induction, the experiment was assayed on nonhost tobacco and tomato. Bacterial suspension was separatly prepared from cell cultures of Xag mutant and wildtype strains with sterile distilled water as method described above. Bacterial suspensions at various cell density of about $2x10^8$, $5x10^9$, and $2x10^{11}$cfu/ml were individually or mixed together with 10% glucose (v/v) were infiltrated into the mesophyll of each tested plants which 4 to 5 weeks old of tobacco, and 3 or 4 weeks old of tomato respectively by using a 1-ml hypodermic syringe without a needle (Wei *et al*., 1992). The inoculated plants were maintained in greenhouse at 30^oC and high relative humidity. Infiltrated areas were monitored for development of tissue collapse and necrosis for 24-48 h post infiltration

10. Assays of extracellular enzymes and exopolysaccharide production

10.1 Bacterial culture conditions

The phenotypic defection of Xag mutant of extracellular enzymes production was established. Xag wildtype, mutant, and complemented strains were individual cultured on LB plate for 24 – 48 h at room temperature (28-30^{0}C). One loop of each strain was removed into 5 ml of LB broth contained in glass tube and incubated on rotary shaker at 150 rpm at room temperature for over night (18 h) as stock culture. The 0.5% (V/V) of each bacterial suspension was added in tested medium, M9 medium (0.5 g of NaCl, 1 g of NH_4Cl, 3 g of KH_2PO_4, 7.5 g of Na_2HPO_4 x $2H_2O$, 4 g of glucose, 120 mg of $MgSO_4$, and 10 mg of $CaCl_2$, and water contained in total volume of 1000 ml sterilized distilled water). Bacterial culture was incubated at room temperature on rotary shaker for 24 h. Cell suspension in liquid culture at log stage was precipitated by refrigerator centrifuge at 8,000 rpm for 15 min at 4 ^{o}C. The supernatant obtained was purified by sterilized syringe filter. Each supernatant was kept in low temperature at 4^{O}C until used for analysis.

10.2 Cellular fractionation

Cultured media of Xag wild type strain were centrifuged at 8,000 rpm for 10 min. The supernatant was kept as the extracellular fraction using in quantitation of exoenzyme assay. The pellet was washed twice with equal volumes of water and then treated on ice for 2 h with lysozyme (200 mg/ml) in a solution made up of 20% sucrose, 30 mM Tris-HCl (pH8.0), and 1 mM EDTA. The lysozyme- treated cells were pelleted by centrifigation at 10,000 rpm for 10 min. The supernatant was collected as the periplasmic fraction. The cell pellet was resuspended in 10 mM Tris-HCl (pH 8.0), passed through a 24-gauge needle, and centrifuged at 12,000 rpm for 15 min. The supernatant was collected as the cytoplasmic fraction (Hu *et al*., 1992. Each of the above fractions was precipitated by 50 % (wt/vol) ammonium sulfate and centrifuged at 8,500 for 10 min. The pellets were dissolved in one-tenth the original volume of acetate buffer and assayed for enzyme activity as described below.

10.3 Enzyme plate assay

The production of extracellular enzymes included cellulases, proteases and alpha-amylase were investigated as method described by Ray (2002) with some modifications as Andro *et al.* (1984) as followed.

10.3.1 Cellulase plate assay

The 30 µl of clarified supernatant was droplet onto the 0.5 cm diameter hole of PSA plates containing 0.5 % carboxy methyl cellulose (CMC ; Sigma chemical) and incubated at room temperature. A white halo surrounding the colonies against the blue black ground of the plate indicated secretion was determined at 48 h after incubation with a 1% (wt/vol) Congo red (Sigma) for 15 min and destained with 1 M NaCl solution for 15 min.

10.3.2 Alpha-amylase plate assay

The reference alpha-amylase test was performed as a starch hydrolysis procedure. Starch plates agar were prepared and used within 3 weeks. 30 µl of clarified supernatant was droplet onto the 0.5 cm diameter hole on starch plate and incubated for 24 h. After incubation, iodine solution was added to the surface of the plate, with positive demonstrating a zone of clearing around the inoculum.

10.3.3 Protease plate assay

Protease activity was determined by an agar plate assay. The test agar contained 1% skim milk, 1% tryptone (Difco), 0.5% yeast extract (Gibco BRL), 0.5% NaCl, and 1.5% agar. The 30 µl of clarified supernatant was droplet onto the 0.5 cm diameter hole on protease test plate and incubated for 48 h. A white halo surrounding the hole in tested plate indicated secretion was determined as positive reaction.

10.4 Quantification of exoenzymes

Quantitation of exoenzyme in different cellular fractions was done according to the procedure described by Biely *et al.* (1988). One ml individual sample cellular fraction of each strain was separated from stored in test tube fixed on ice box (4^oC). The 3 ml of dinitrosalicylic acid (DNS) reagent was added into 3 ml of glucose solution sample in a lightly capped test tube. To avoid the loss of liquid due to evaporation, cover the test tube with a piece of paraffin film if a plain test tube is used. Then, each sample tube was heat the mixture at 80º C for 15 min to develop the red-brown color. Add 1 ml of a 40 % potassium sodium tartrate (Rochelle salt) solution to stabilize the color. After cooling to room temperature in a cold water bath, recorded the absorbance with a spectrophotometer at 575 nm.

10.5 Enzyme and toxin activity assay

All of tested bacterial strains, *X. axonopodis* pv. *glycines* wildtype mutant and complemented strains were cultured in general medium and induced media, carboxy methyl cellulose medium (CMC) plate for 48 h. One loop of each strain was moved in 5 ml CMC broth and LB broth contained in glass tube and incubated on rotary shaker at 150 rpm at room temperature for over night, after that 0.5 % (V/V) of each cell bacterial suspension was added in 250 ml CMC-broth and LB broth contained in 500 ml flask size and incubated at room temperature on rotary shaker for 72 h. Cells cultured were harvested and precipitated by refrigerator centrifuge at 8,000 rpm for 15 min at 4^oC. The supernatant separated by sterilized syringe filter and precipitated by 50% (wt/vol) ammonium sulfate and centrifuged at 8,500 rpm for 10 min. The pellets were dissolved in one-tenth the original volume of acetate buffer and assayed for phytotoxic activity as method applied follow.

To assess phytotoxicity of extracellular enzyme, leaves of approximately 14 days old of susceptible soybean SJ4 cultivar was tested. Enzyme solution obtained from previous step was diluted with sterilized distilled water (SDW) as ratio (cellulase: SDW) of 1:1, 1:2, 1:3, 1:4, and 1:5 respectively. The 10 ul of each

concentration was applied into mesophyll of soybean plants by infiltration with 1-ml syringe with out needle or as a 5 ul droplet after slightly puncturing the leaves with a needle. After 24, 48, 72, and 96 h of incubation, the tested leaves were monitored for appearance of necrotic lesions.

10.6 EPS extraction

For measurement of EPS production, Xag wildtype, mutant, and complemented strain were cultured separately in tryptone-glucose yeast (TGY) broth containing (per liter of distilled water) 5 g tryptone, 5 g glucose, 3 g yeast extract, 700 mg K_2HPO_4, and 250 mg $MgSO_4 \cdot 7H_2O$ was used. A loop of 48-h-grown culture of each bacterial strain was inoculated into 20 ml of TGY broth, and the culture was allowed to grow for 48 h at 28 °C using a rotary shaker at 180 rpm. A 2% inoculum was then transferred into 250 ml of TGY broth and allowed to grow for 120 h at 28 °C in a rotary shaker at 180 rpm. EPS was precipitated from culture supernatants by ethanol, dried, and weighed as described by Tang *et al.* (1991). Cultured was centrifuged at 7,000 rpm for 10 min at 4 °C to remove the cells. The supernatant was mixed with a 10% (v/v) saturated KCl solution as an electrolyte, precipitated with an equal volume of 95% ethanol, and kept at 4 °C overnight. The precipitate was removed by centrifugation at 8,5000 rpm for 15 min at 4°C, washed twice with 95% ethanol, air-dried, and measured quantity by weighting analysis.

11. Assay of Systemic resistance induction on soybean plants

The repesentative mutant strain was used in the induction of defense reactions against pathogenic Xag wildtype. The experiments included these following.

11.1 Plant materials

The pot experiment was conducted in greenhouse conditions. Soybean seedlings were sown from seeds of susceptible SJ4 cultivar and grown as needed in plastic pots of 8 inch diameter, 5.5 inch height, 30 cm^3 soil volume; containing sterile

loamy organic soil mixtures without supplemented lighting. Soil water content was regulated to standard protocol. Plants were fertilized weakly with a modified Hoaglands nutrient solution (pH 6.5). The temperature in the greenhouse was maintained between 29-35 ^{O}C (night to day). The humidity in the green house was around RH 70% appropriate stage of soybean plant at 14 day old was selected for experiment.

11.2 Assay for detected defense responses

The analyze ability of Xag *ppsA* mutant strain KUMNTP2 on host defense induction against pathogenic Xag, wildtype was performed with two set. One set of plants was singly treated with Xag wildtype mutant, and complement strains. The another set was challenge inoculated on tested soybean plants with wildtype and complement strains at 24 h after treated with mutant. Cell suspensions of each Xag strain were prepared with sterile distilled water at the optimum density of 10^8 cfu/ml and sprayed onto the 14-day old susceptible soybean SJ4 cultivar. Foliar spray plants with sterile distilled water (SDW) that neither treated with mutant nor challenged by the pathogen wildtype were used as a negative control. Sampling was collected at 1 day interval to 7 days after challenge inoculation with wildtype for analysis of defense-related enzymes included total phenolic compounds, phenylalanine amonialyases (PAL)**,** peroxidase (POX), and *β*-1,3-glucanase as described in the following sections. The mean area of the lesions in infected leaves was expressed with the method described by Prathuangwong *et al.* (1993). The experiment was designed by completely randomized design (CRD) on a greenhouse bench, each treatment consisting of five replications; each replicate comprised with 5 pots; in each pot 3 plants was maintained; and conducted twice.

12. Detection of phenolic compounds and activities of defense-related enzymes

12.1 Phenol assay

Soybean leaves were carefully collected 1, 2, 3, 4, 5, 6, and 7 days after pathogen challenge inoculation. Soybean leaf samples (1g) of treated seedlings were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70 °C. One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteau reagent (1N) and the solution were kept at 25°C. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. Catechol will be used as the standard. The amount of phenolic was expressed as µg catechol mg^{-1}protein (Zieslin and Ben–Zaken, 1993).

12.2 Total protein extraction and quantification assay

Protein assay was performed on soybean leaf extracts collected at 1 to 7 days after pathogen challenge inoculation. Leaf tissue extracts of 0.1 g fresh weight were grounded in a mortar and pestle containing extraction buffer [0.1 M Tris–HCl buffer, pH 7, 0.1 M KCl, 1 mM PMSF, 1 % (v/v) Triton X–100, 3% (w/v) PVPP]. The extract was filtrated through cheese doth and the filtrated was centrifuged at 16,000 g for 15 min. The supernatants were kept in an ice box and used for determination of PAL**,** POX and *β*-1,3-glucanase activities and for dosing total protein (Thipyapong *et al.,* 2004).

12.3 Estimation of PAL activity

PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Sample containing 0.4 ml of soybean protein extract was incubated with 0.5 ml of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The amount of trans-cinamic acid synthesized was calculated using its extinction coefficient of 9630 m^{-1}.

Enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} mg^{-1} protein (Ramamoorthy *et al*., 2002).

12.4 POX assay

Peroxidase activity was determined at 30°C by a direct spectrophotometric method. The reaction mixture consisted of 10 ul soybean leaf protein extact and 1019 µl of a solution containing guaiacol, hydrogen peroxide and sodium phosphate buffer (125 ul guaiacol+153 µl hydrogen peroxide in 50 ml of 10 mM sodium phosphate buffer, pH 6.0). The reaction was incubated in a water bath and absorbance readings at 460 nm were taken every 30 seconds for 15 and one half minutes. Peroxidase activity was expressed as absorbance units min^{-1} mg $protein^{-1}$ (Hammerschmidt *et al.,* 1982).

12.5 β -1,3-glucanase assay

β-1,3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method. The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of soybean leaf protein extract. The reaction was carried out at 40°C for 10 min. The reaction was then stopped by adding 375 µl of dinitrosalicylic acid and heating for 5 min on a boiling water, vortexed and its absorbance was measured at 500 nm. The enzyme activity was expressed as µg glucose released min^{-1} mg^{-1} protein (Pan *et al.,* 1991).

13. Bacterial growth in soybean leaves and disease suppression by Xag mutant strain

The competitive index defined as the change in the population ratio of Xag strains after inoculated in plant tissues under greenhouse conditions with susceptible soybean cultivar SJ4 prepared as methods described above**.** Bacterial growth and disease induction was conducted by inoculation bacterial suspension into soybean leaves by foliar spray and tissue infiltrated. For bacterial multiplication tested, ten µl of each inoculum of Xag wildtype, mutant, and complement strains at 10^8 cfu/ml was used in single and co-inoculations by tissue infiltration in soybean leaves at 24 h post-

foliar sprayed with mutant strain. Bacterial populations of each Xag strain in each treatment were detected at infiltrated area at 1 to 5 days after infiltration for quantification of bacterial titer. Samples were macerated and placed individually in 1 ml of sterile distilled water, triturated, and isolated by plating for individual colonies on YP medium containing the appropriate antibiotics including 50 µg/ml rifampicin, 50 µg/ml kanamycin, and 50 µg/ml of tetracycline for isolated wildtype, mutant, and complement strains respectively.

The foliar spray inoculation with the 20 ml of mutant suspension was pre-treated leaves on tested soybean plants and kept in greenhouse conditions at 1, 2, 3, and 4 days before challenge with wildtype. The pathogenicity of each strain on inoculated soybean plants was performed and observed at 7 and 14 days after inoculation (Prathuangwong *et al.,* 1993). The individual cell suspension of wildtype and mutant strains were separately sprayed onto soybean leaves used as a positive and negative control were included in each trial respectively. The decrease in population density *in planta*, and suppression of the disease severity were considerate as induced plant resistant strategies. Growth of mutant and the associated strains together with their expression of disease index were assayed twice with qualitatively similar results**.**

RESULTS AND DISCUSSION

Results

1. Isolation and Characterization of *Xanthomonas axonopodis* pv. *glycines*

All of the 10 strains of *Xanthomonas axonopodis* pv. *glycines* (Xag) were originated from different soybean production areas of Thailand **(**Table 2). Most strains were isolated and identified by Saiseangthong (1999) and classified in virulent group with phenotype characteristics and rep-PCR by Ketsuwan (2003) accepted strain No.12-2 was isolated in this study. The pathogenicity of these bacterial strains was re-evaluated using foliar spray inoculation on different soybean cultivars. The incident and severity of bacterial pustule on tested plant was determined by Prathuangwong et al. (1993) at 7 and 14 days after inoculation. All of Xag strains could induce different level of disease severity depend on bacterial strain and host cultivar. Susceptible soybean cultivars SJ4, SJ5, and CM60 were exhibited ranging disease severity from 35 to 78% whereas the resistance cultivar ST1 showed a lightly disease severity (Table 3). Xag strains BK01, No.12-2, RE08, No.21, and CM60-01 revealed high ability to induce disease severity on soybean SJ4 cultivar with 75, 73, 70, 68, and 65 respectively. All of 5 virulent strains were then used for the representative pathogenic strains determined in the next step.

Table 3 Ability of *X. axonopodis* pv. *glycines* (Xag) different strains to induce bacterial pustule disease symptom on different soybean cultivars

Xag strain	Code of strain	Disease severity (%) [1/]			
		Soybean cultivar			
		SJ 4	SJ 5	CM 60	ST 1
KU-K-46012	No.12-2	73 a	75 a	35.6	2 ab
KU-K-44065	NKR13	45 d	54 d	15.20	2 ab
KU-K-44069	NKR17	58 c	57 d	16.23	1 b
KU-K-44072	NKR21	60 b	62 c	35.4	3 a
KU-K-44102	NO.15	48 d	47 e	22.6	2 ab
KU-K-44105	NO. 21	68 ab	65 b	41.24	2 ab
KU-K-45116	RE07	60 b	62 c	41.20	1 b
KU-K-45117	RE08	70 a	71a	38.36	2 ab
KU-K-45147	CM60-1	65 ab	67 b	39.8	1 b
KU-K-44089	BK01	75 a	78 a	39.65	3 a
CV.	-	12.5	10.4	5.8	5.6

[1/] Disease severity was expressed as percent leaf area appear of disease symptom infection with Xag evaluated at 7 days after inoculation by the method of Prathuangwong (1993). Means in the column followed by the same letter are not significantly different ($p < 0.05$) according to Duncan's New Multiple Rang Test.

The representative strains of Xag including BK01, No.12-2, RE08, No.21, and CM60-1 which high abilility to induce disease severity were selected for testing HR induction on nonhost, tobacco (*Nicotiana tabacum* cv. Xanthi,), pepper (*Capsicum annuum* cv. Cayenne), and tomato (*Lycopersicon esculentum* cv. Seedatip-II). All of bacterial strains showed different typical HR symptom with necrotic lesion on tested plant leaves depend on plant specie and Xag strain. Pepper and tomato showed highest sensitive responded at 18 h after infiltration with 0.5 OD (A_{600nm}) of each suspension whereas tobacco showed clearly HR at 24 h after infiltration (Table 4). Bacterial strains BK01 and N0.12-2 were high consistently HR expression on tobacco leaves (Table 4).

Then, the minimum concentration of each Xag strains on HR induction was observed by serial cell concentrate at $2x10^8$, $5x10^9$, $5x10^{10}$, $5x10^{11}$ cfu/ml as method described above. The results showed the concentration of each Xag suspension at $5x10^{11}$ cfu/ml (OD=1.5) was extremely elicited HR on tobacco which produced rapid localized death of plant cells with no spread of the bacteria to surrounding tissues. The affected areas were found to occurre the first signs of necrosis in a very short time within 16-24 h after infiltration. Three to four days after death of host cells, the injected areas became dry and white same as previously reported (Kaewnum, 2005). The complete collapse of infiltrated tissue was not observed on tobacco leaf when infiltrated suspension concentration was less than $5X10^9$ cfu/ml (OD = 1.0) excepted the strains No12-2 and BK01 that could induce HR symptom with lower cell concentration at $2x10^8$ cfu/ml (Table 5). These result indicte that the minimal concentration of Xag needed to induce the HR on tobacco was approximately at $5x10^9$ cfu/ml and depend on bacterial strain whereas some aggressiveness strain could induce the HR with lower cell concentration. Therefore, these results revealed that Xag strain BK01 and No.12-2 show highest disease severity and clearly HR induction, both of these strains were selected for mutagenesis and study on their pathogenicity related genes in the next step.

Table 4 Ability of *X. axonopodis* pv. *glycines* to cause the HR on different tested nonhost plants infiltrated with 0.5 OD_{600} of cell suspension

Xag strain	HR reaction on tested plant[1/]		
	Tobacco	Pepper	Tomato
No.12-2	+	+	+
NKR13	-	+	+
NKR17	-	+	+
NKR21	-	+	+
NO.15	-	+	+
NO. 21	-	+	+
RE07	-	+	+
RE08	-	+	+
CM60-1	-	+	+
BK01	+	+	+

[1/] HR= hypersensitive response ; + = positive reaction, - = negative reaction. Infiltrated areas were monitored for development of tissue collapse and necrosis within 48 h post inoculation. The positive reaction was complete collapse and necrosis of the entire infiltrated area and the negative reaction was no visible lesion. Optical density (OD, $A_{600\ nm}$) of 0.5 was accorded to cell density of $5x10^9$cfu/ml respectively.

Table 5 Ability of *X. axonopodis* pv. *glycines* to cause the HR on tobacco leaves infiltrated with various cell concentrations

Strain	HR reaction [1/]			
	Cell concentration (OD, $A_{600\ nm}$)			
	0.2	0.5	1.0	1.5
No.12-2	+	+	+	+
NKR13	-	-	+	+
NKR17	-	-	-	+
NKR21	-	+	+	+
NO.15	-	-	-	+
NO. 21	-	-	+	+
RE07	-	-	-	+
RE08	-	-	+	+
CM60-1	-	-	+	+
BK01	+	+	+	+

[1/] HR= hypersensitive response ; + = positive reaction, - = negative reaction. The negative reaction was no visible reaction. The positive reaction was complete collapse and necrosis of the entire infiltrated area. Infiltrated areas were monitored for development of tissue collapse and necrosis within 48 h post inoculation. Optical density (OD, $A_{600\ nm}$) at 0.2, 0.5, 1.0 and 1.5 accorded to cell density of $2x10^8$, $5x10^9$, $5x10^{10}$, $5x10^{11}$ cfu/ml respectively.

2. Transposon mutagenesis

To random isolation the pathogenicity associated genes of Xag, the transposon mutagenesis was used for insertion into chromosome of Xag strains BK01 and No.12-2 by triparental conjugation mating, with donor Tn5 pSUP2021 and pJB4JI. The 2,580 colonies of transconjugants were obtained on YP agar supplemented with 50 ug/ml kanamycin. All of mutant strains were obtained form random Tn5 inserted into Xag strain No. 12-2, where as strain BK01 could not screened any colony from this method (data not shown). This result indicated that transfer frequencies of Tn5 fragment were influenced to a large extent by the mating conditions and physiological state of donor and recipient cells investigate. All of transconjugant colonies were individual purified by twice cross streak on YP medium plate supplemented with 50 ug/ml kanamycin before the phenotype investigated.

3. Screening of Xag mutants

3.1 Ability on disease induction

Following treatment of Xag mutant strains with Tn5 mutagenesis, the total of 2,580 transconjugant colonies were tested for their pathogenicity deficiency on host plant, susceptible soybean cultivars (SJ4) with cotyledon assay, and foliar spray inoculation. The cotyledon bioassay data for mutant and their pathogenicity are shown in Table 6, Fig 2, and Fig 3. Among the mutants obtained, they could exhibited only four transconjugant colonies namely KUM00812, KUPJ124, KUMNTP2, and KUM408 were apparently deficient of the pathogenicity on tested cotyledon within 24 h, as revealed by the absence of necrotic symptom. In some case, minor chlorosis was noted around the inoculation site, but this also occurred on negative controls by using sterilize distilled water. There was no evidence of water soaking or intarcellular growth of the mutants (Fig. 2). Only three mutant strains including KMPJ124, KMPS408, and KUMNTP2 were apparent clearly deficient pathogenicity. The strain KUMNTP2 revealed by the absence of necrotic lesion on inoculation site on tested cotyledon, excepted strains KMPS124 and KM408 showed the minor chlorosis around

the inoculation site at 48 h after inoculation comparing with wild type No.12-2 which revealed clearly necrotic symptom and cell collapse on cotyledon with in 24 and 48 h after inoculation respectively. Therefore, all of four mutant strains were then tested for the pathogenicity on host plant by foliar spray inoculation. The results revealed only mutant strain KUMNTP2 showed complete deficient pathogenicity on both of cotyledon and foliar bioassays (Fig.2 and Fig. 3) when detected at 7 days after inoculation, whereas wildtype strain, No.12-2 and BK01 showed severe disease symptom on tested plant at 14 days after inoculation of 75 and 72% disease severity respectively (Table 6). Typical symptoms of bacterial pustule of young greenish-pale spots caused by wildtype was occurred with in 4 days and showed clear symptoms of necrotic spot appeared slightly raised and developed into a small pustule on the underside of the leaves and produced a large, yellow to brown area with small, dark brown spots at 7 days after inoculation. The representative mutant strain KUMNTP2 was failed to induce disease symptoms on cotyledon and tested host plants in 7 days. In additional, the strain KUM408 showed different typical symptom on soybean leaves while the other two strains, KUM00812, and KUPS124 showed some lesions on soybean leaves with 30 and 25% disease severity at 14 h after inoculation.

Table 6 Efficacy of *X. axonopodis* pv. *glycines* mutants and wild type strains for induced bacterial pustule symptom on susceptible soybean SJ4 cultivar and hypersensitive response on different nonhosts

Strains	No. of chlorotic cotyledon/ no. tested [1/]	Disease severity (%)[2/]
Mutant strain		
KUPJ124	15/30	25
KUMNTP2	5/30	0
KUMP408	6/30	15
KUM00812	10/30	30
Wildtype No.12-2	30/30	75
E.coli DH5∝ (Negative control)	0/30	-

[1/] Disease incidence subjected to infiltration method monitored for development of tissue collapse and necrosis that + = slightly necrotic spots visible in infiltrated area ; ++ = moderated necrosis at margins of infiltrated area ; +++ = clear necrosis of the entire infiltrated area with brown tissue; ++++ = completely dried at infiltrated area ; and - = no visible spot was observed .

[2/] Diseases severity subjected to foliar spray method expressed as percent leaf area infection as described by Prathuangwong *et al.* (1993).

[3/] HR= hypersensitive response ; + = positive reaction, - = negative reaction, infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h post inoculation. The negative reaction is no visible necrosis. The positive reaction is complete collapse and necrosis of the entire infiltrated area.

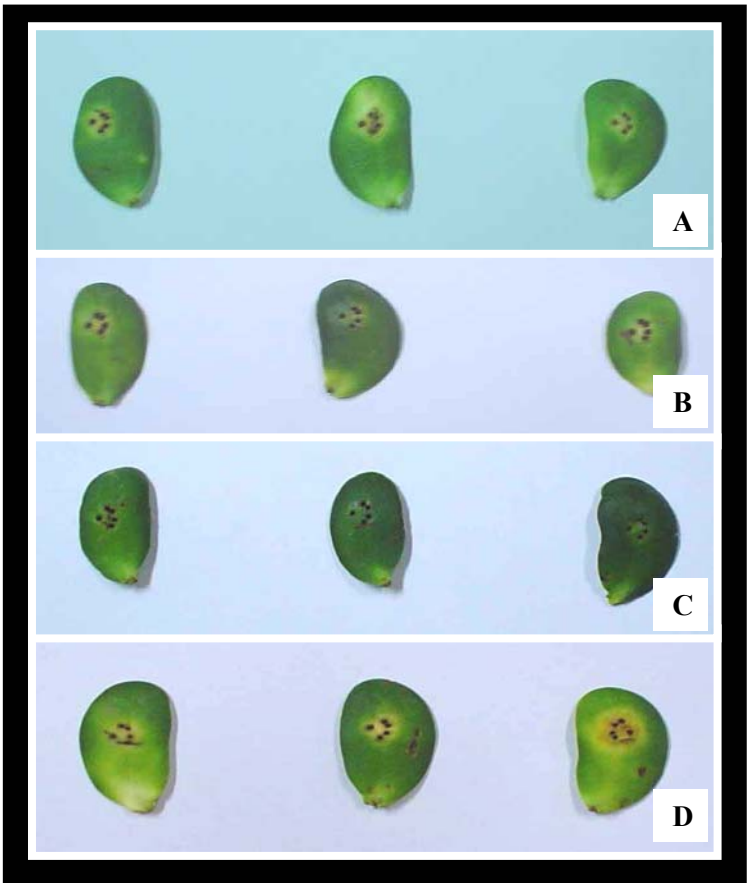

Figure 2 Pathogenicity test using a cotyledon bioassay for pathogenicity minus strain screening. Three mutant strains including KMPS124 (A), KMPS408 (B) and KUMNTP2 (C) were apparently deficient pathogenicity, as revealed by the absence of necrotic on inoculation site of cotyledon excepted strains KMPS124 and KM408 showed the minor chlorosis around the inoculation site at 48 h after inoculation (yellow arrow) comparing with wildtype No.12-2 (D) which reveale clearly necrotic symptom on cotyledon with in 24 h after inoculation.

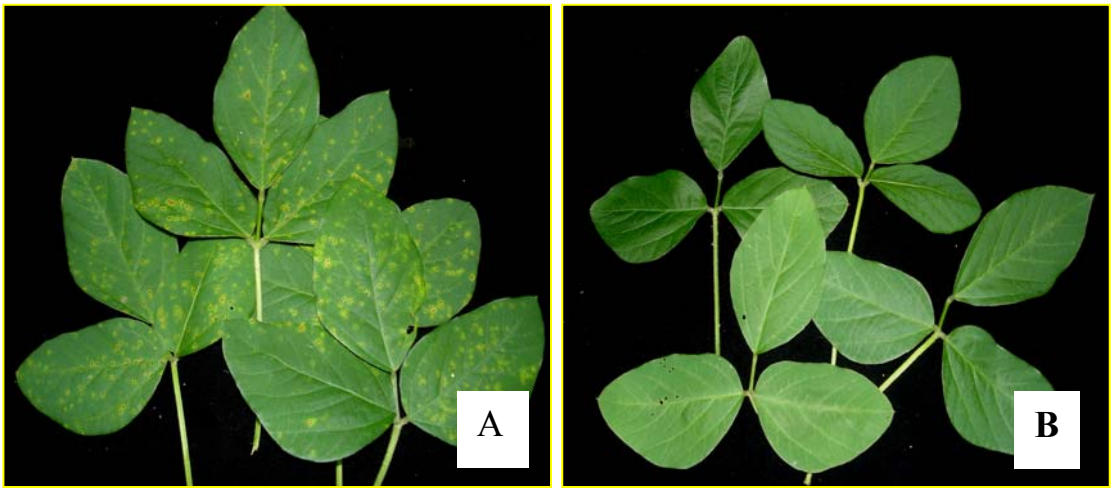

Figure 3 Disease symptom on soybean leaves at 14 days after spray inoculation with different bacterial suspension. Wildtype strain (A) show high disease severity on soybean leaves, where as mutant strain (KUMNTP2) could not induce disease on tested plant (B).

3.2 Characterization on HR induction on nonhost and host plant

The preliminary tested on hypersensitive response on nonhost plant revealed that all of Xag mutant strains KUM00812, KUPJ124, and KUM408 including wildtype No12-2 could induce HR on nonhost plant tobacco and tomato excepted the KUMNTP2 which able to induce HR on tomato but not on tobacco when the cell suspension at 0.2 OD was infiltrated (Table 7 and Fig.4). The results suggested that the deficient pathogenicity of mutant should be occurred by Tn5 insertion attached genes involved their pathogenicity in different site on chromosome of Xag strains. Moreover, all of mutant strains have shown the different characteristic of their pathogenicity and hypersensitive response. The evident effect could be elucidat that each mutant strains were inserted in different site and position of pathogenicity related genes and affected to different mutagenesis phenotype. Therefore, the ability of plant pathogenic bacteria to cause disease in particular host-plant depends on several factors especially the pathogenicity of plant pathogenic bacteria including Xag can be expressed through several infection stages, invasion, recognition, multiplication of bacterial cell, production of virulence factors, and symptom development. These stages are involved to the genetic controlling. However, as the result shown that only mutant strain KUMNTP2 was deficient or delayed pathogenicity and unable induced HR on tobacco. Therefore the mutant strain KUMNTP2 was further selected as the representative strain for pathogenicity related gene characterization.

3.3 HR induction on reistant soybean cultivar

As the result reported above, the representative mutant KUMNTP2 and wildtype strain were continue tested for HR induction on the resistance cultivar of soybean (ST1) using various cell concetraion at 0.2, 0.5 and 1.0 OD (A_{600}). The results showed that both of wildtype and mutant KUMNTP2 could induced the HR lesion on tested plant within 24 h after infiltration (Fig.5). There were not different ability to induced HR symptom when infiltrated by using different cell concentration at 0.2, 0.5 and 1.0 OD (A_{600}) (Table 8). The initial symptom of HR occurred on resistant soybean leave exhibited as small water-soaking spot around the infiltrate area with in 24 h. The

necrotic lesion was rapid developed within 48 h post infiltration and the size of HR symptom was limited, not spread to beside iffiltrate area (Fig.5). Three to four days after death of host cells, the injected areas became dry and white same as HR on non host plant.

The results suggested that the deficient pathogenicity of mutant strain KUMNTP2 migh be affected by Tn5 insertion within only gene involved in the pathogenicity or virulent factor, but not direct to disrupted on the hypersensitive response–related genes encoded by *hrp* regulation and involved in the host-pathogen interaction. Moerove, the population density of bacterial cell or its ability to multiplication in host-plant mignt be not effect to HR induction. Therfore, many reports were suggesting that the pathogenicity and virulence of phytopathogenic bacteria are related in several factors. Thus, the pathogenicity properties Xag among wild type and mutant strains have been done in the next step.

Table 7 Efficacy of *X. axonopodis* pv. *glycines* mutants and wildtype strains for hypesensitive response on different nonhosts, tobacco and tomato at 48 h after cell concentration at 0.2 OD was infiltrated.

Strains	HR induction[1/]	
	Tomato	Tobacco
Mutant strain		
KUPJ124	+	+
KUMNTP2	+	-
KUMP408	+	+
KUM00812	+	+
Wildtype No.12-2	+	+

[1/] HR= hypersensitive response ; + = positive reaction, - = negative reaction, infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h post inoculation. The negative reaction is no visible nercrosis. The positive reaction is complete collapse and necrosis of the entire infiltrated area.

Table 8 Hypersensitive response induced by *X. axonopodis* pv. *glycines* wildtype and mutant strain KUMNTP2 on resistance soybean cultivar ST1.

Cell concentration (OD. at $A_{600\ nm}$) [1/]	HR induction [2/]				
	Induction period (h)				
	12	24	48	72	96
0.2	-	+	++	++	+++
0.5	-	+	++	+++	+++
1.0	-	+	++	+++	+++

[1/] Cell concentration at 0.2, 0.5 and 1.0 OD (A_{600}) accorded to cell density of $2x10^8$, $5x10^9$, $5x10^{11}$ cfu/ml respectively

[2/] HR= hypersensitive response ; + = positive reaction, - = negative reaction ; Infiltrated areas were monitored for development of tissue collapse and necrosis. The negative control is no visible reaction. The resulting reactions were given numeric values, - = no visible reaction; + = slightly necrotic spots visible in infiltrated area; ++ = moderated necrosis at margins of infiltrated region; +++ = clearly necrosis of the entire infiltrated area with brown color. And ++++ = complete necrosis suroudbing infiltrated area.

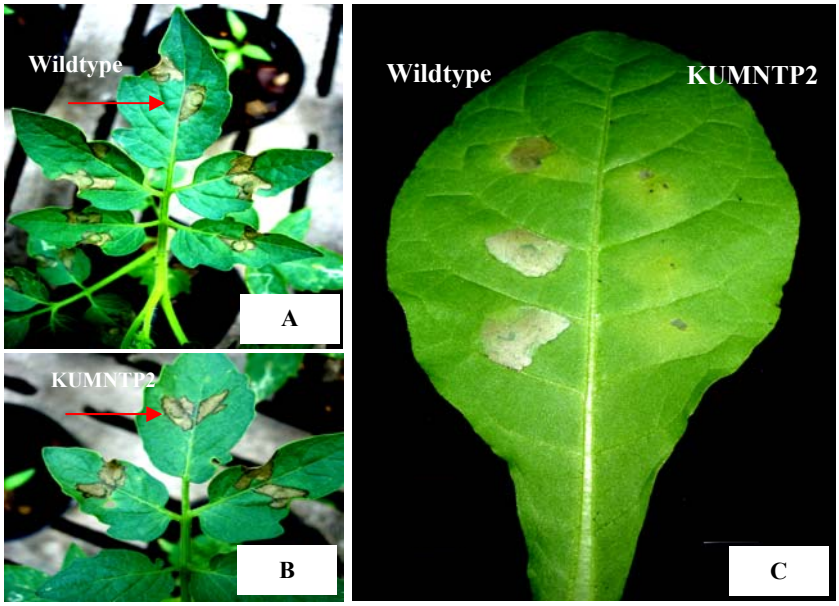

Figure 4 Hypersensitive response (HR) tested, wildtype strain No.12-2 show clearly typical HR both on tobacco (A) and (C) tomato all of cell concentration , whereas the mutant strain KUMNTP2 retained the ability to induce an HR on tomato but not on tobacco at cell concentration of 10^8 cfu/ml (B) and (C).

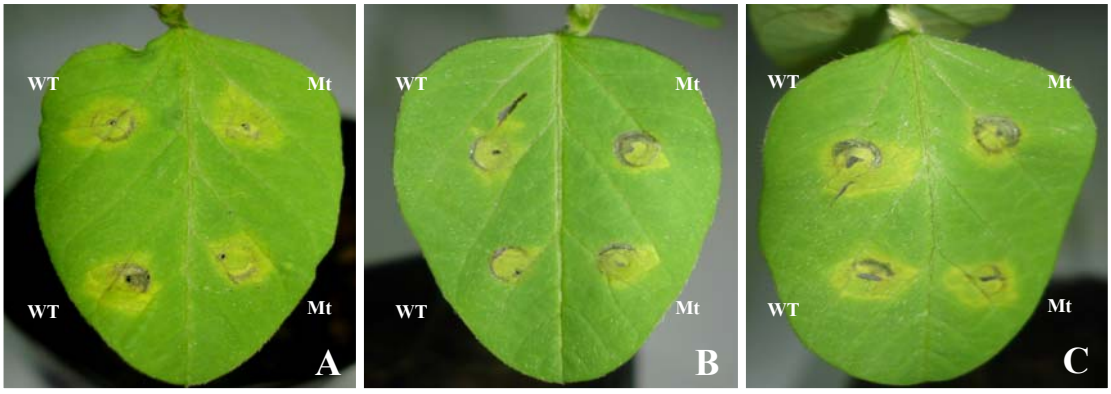

Figure 5 Hypersensitive response induced by *X. axonopodis* pv. *glycines* wildtype (WT) and mutant strain KUMNTP2 (Mt) on resistance soybean cultivar ST1. The initial symptom of HR on resistant soybean leave exhibited as small with in water soaking spot around the infiltrate area 24 h after infiltrated with different cell concentration at 0.2 (A), 0.5 (B) and .0 (C)

5. Identification of the mutated gene

The Tn5 fragment inserted in the mutant chromosome was analysis. A total genomic DNA of bacterial mutant strains KUMNTP2, KUM408, and KUM 124 were isolated by chloroform extraction and ethanol precipitations (Chen and Kuo, 1993). The transposition site in chromosomal DNA of mutant was preliminary determined by PCR analysis using Tn5/F and KAN2/R primer mentioned in the materials and methods. The results revealed that the 1.1 kb DNA fragment of transposition was detected from chromosome fragment of mutant strains KUMNTP2, where as absent in wildtype strain (Fig. 6). This result confirmed that the deficient pathogenicity of mutant strain has been disrupted by Tn5 insertion. The 1.1 kb fragment was also detected from other 2 mutant KMPS124, and KUM408 (Fig. 6). They were however, inserted with Tn5 in different site and showed different pathogenic assays compared with wildtype strain

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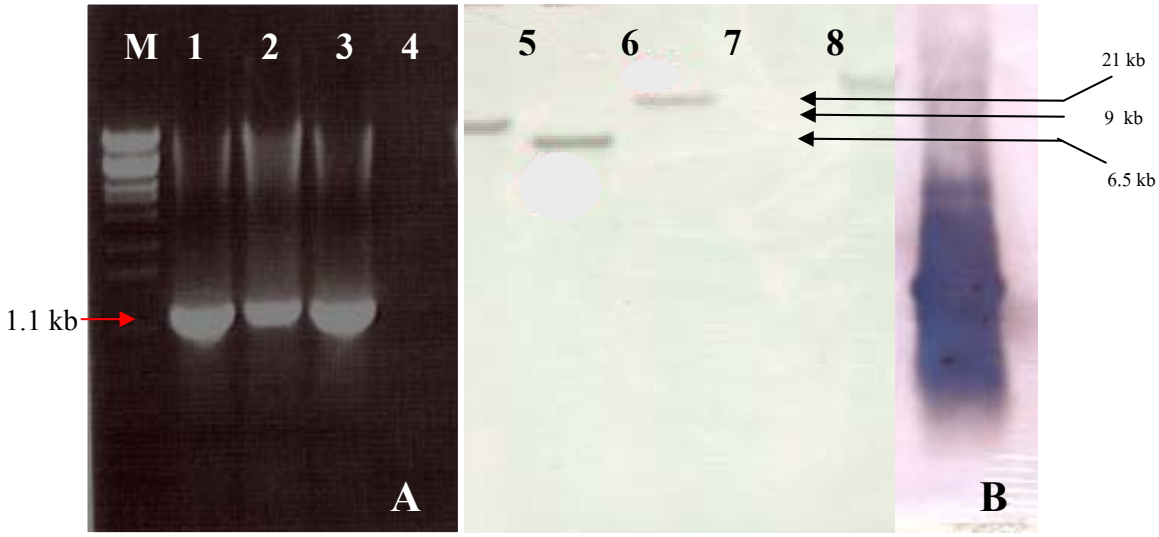

Figure 6 Analysis of Tn5 insertion by PCR (A) and Southern hybridization (B). PCR product were amplified by used Tn5-Km^R primers, presented the 1,100 bp. The results revealed that the DNA fragment of transposition was detected from chromosome of mutant strains KUMNTP2, KUM408, and KUM 124 (lanes 1,2 and 3 respectively), while absent in genome of wild type strain (lanes 4). This confirms that the deficient pathogenicity have been disrupted by Tn5 insertion. Southern hybridization have shown the different nserted position of Tn5 in each mutant EcoRI fragment at 9, 6.5, and 21 kb for KUM124, KUM408 and KUMNTP2 (lanes 5, 6 and 7) respectively compared with fragment genome wild type (lane 8) and original Tn5 fragment from pSUP2021 (lane9) as negative and positive control, respectively.

6. Cloning and sequencing

The chromosomal DNA of target Xag mutant strain KUMNTP2 exhibited deficient pathogenicity contains transposon fragment inserted carry on the large site of 21 kb. DNAs was extracted as methods described by Chen and Kuo (1993), digested with restriction enzyme *Eco*RI and separated by 0.8 % agarose gel electrophoresis. The DNA band at the size of 21 kb revealved in agarose gel were excised and transfered to an eppendorf tube and extracted with AccuPrep® Gel Purification Kit (Bioneer), ligated with pUC118 and transformed in to pUC118 by heath shock technique. Transformants could selected on LB agar containing 100 mg/ml amplicillin and 50 mg/ml kanamycin. The target plasmid containing transposon flanking DNA (pUXMNTP2) could detected by PCR with 1.1 kb fragment size of Tn5/F and Km2/R primer. The PCR product of the appropriate size at 1.1 kb could detected in 0.8 % agarose gel (Fig 7).

Since the 21 kb of *EcoR*I fragment containing target gene inserted was largest and undertaken, subcloned into pBluescript S/K (+) was done as method describes. The smaller target fragments size of 7 kb digested with rectriction enzyme *Sac*I was ligated into pBluescript S/K (+) (Stratagene, La Jolla, CA) and transformed into competent cell *E.coli* strain DH10B by electoporation and selected the target clones on YP agar plate supplemented with 50 ug/ml ampicillin and 50 ug/ml kanamycin. The representative clones form this step was namely pUBLNT2. The plasmid which presumably containing transposon flanking DNA were detected by PCR as methods described above. The flanking region of transposon insertions were sequenced with the syntheic oligonucleotide primer that corresponds to the Tn5 termini and primer specific M13F (5'-AGTCA CGACG TTGTA-3') and M13R 5'-CAGGA AACAG CTATG AC-3') as well as primer walking (Fig.8) with capillary automatic sequencer (Genetic Unit; Shizuoka University).

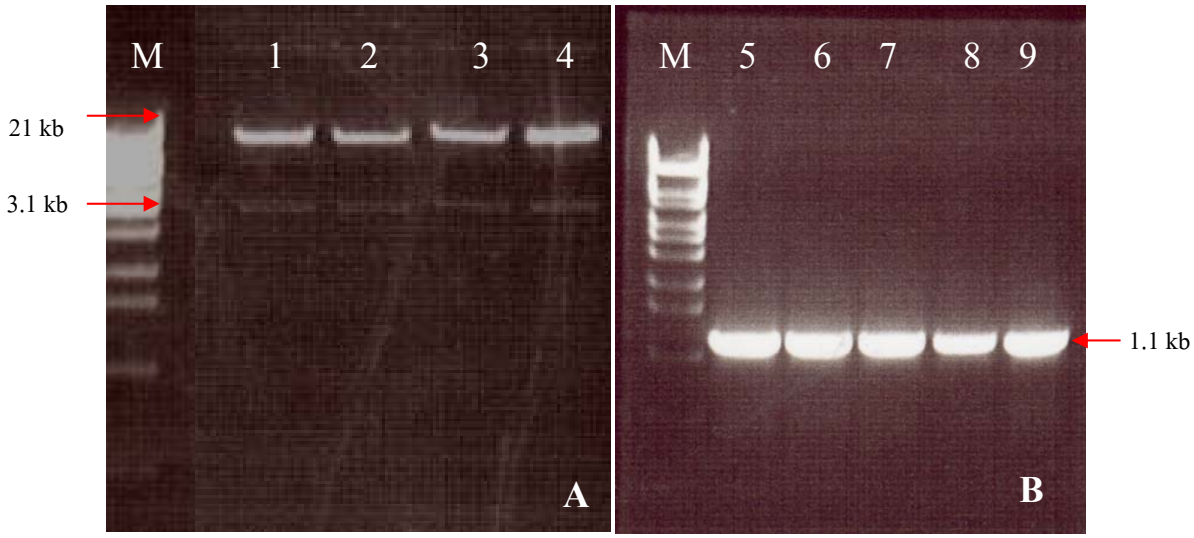

Figure 7 Tn5 fragment inserted of target clone in plasmid vector pUC118 were detected by restriction enzyme (*Eco*RI) analysis (A) which contained 2 fragments of 21 and 3.1 kb as target fragment size and vector size respectively (lanes1-4). All of the representative clones, pUXMNTP2 (lanes 5-9) could be amplified at size of 1.1 kb using Tn5/F and KAN2/R primer (B).

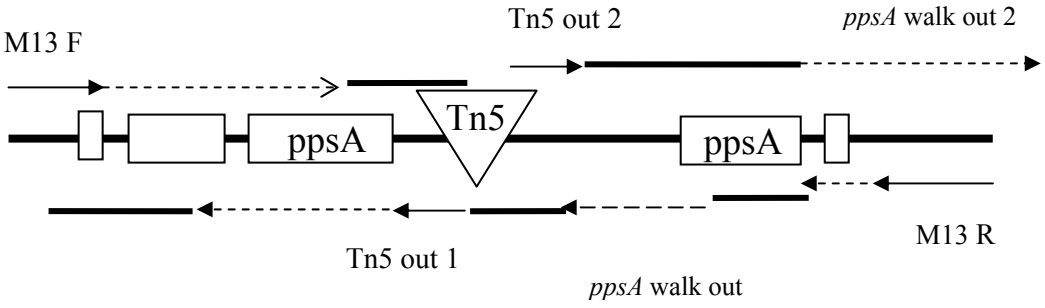

Figure 8 Two-way directions of Tn5 flanking inserted portion on target DNA were used for sequencing analysis. The universal primer M13 F –M13 R and two direction walking primer designed for amplified complete fragment as horizontal arrow show.

Therefore, a 7.1 kb *Sac*I fragment containing a portion of Tn5 (5.8 kb) and Xag flanking DNA was isolated and sequenced with the synthetic oligonucleotide primer that corresponded to the Tn5 termini and primer specific M13F (5'-AGTCA CGACG TTGTA-3') and M13R 5'-CAGGA AACAG CTATG AC-3') and primer walking. The results revealed no Xag sequences similarity to sequences flanking Tn5 insertion. However, sequences were found to be similar to those in phosphoenal-pyruvate synthase (*ppsA*) genes of *X. axonopodis* pv. *citri* strain 360 (GenBank) at 99% (Table 9 and Fig. 9). There was further explored with a BLAST search using possible translation products of larger completed fragment of the homologous among Xag and other *Xanthomonas* spp. The total genes fragment that deficient pathogenicity phenotype KUMNTP2 occurred in a putative gene of 2,374 bp (Fig. 9). The sequences were identified and also showed high significant homolog with *ppsA* of *X. campestris* pv. *vesicatoria* (97%), *X. oryz*ae pv. *oryzae* (94%), and *X. campestris* pv. *campestris* (91%) respectively. The DNA flanking Tn5 was used as a probe to isolate clones from library prepared with genomic DNA from Xag wildtype No.12-2. One of the resulting positive *E.coli* clones was selected for subcloning into pLARF3,a broad-host-range plasmid which low-copy-number for its complementation studies.

Table 9 Nucleotide analysis of *ppsA* and genes of *X. axonopodis* pv. *glycines* mutant strains KUMNTP2 compared with other X*anthomonas* sp.

Tested strain	GenBank (accession number of comparative strain)	Sequence alignment % Identities	Nearest phylogenetic neighbor (genus and species)
KUMNTP2			
	AE011839	99	*X. axonopodis* pv.*citri* str.306
	AM039952	97	*X. campestris* pv. *vesicatoria*
	AP008229	94	*X. oryzae pv.oryzae*
	DQ361034	92	*X. oryzae pv.oryzae*
	AE012313	91	*X. campestris* pv. *campestris*
	AY618213	90	*X. campestris* pv. *campestris*

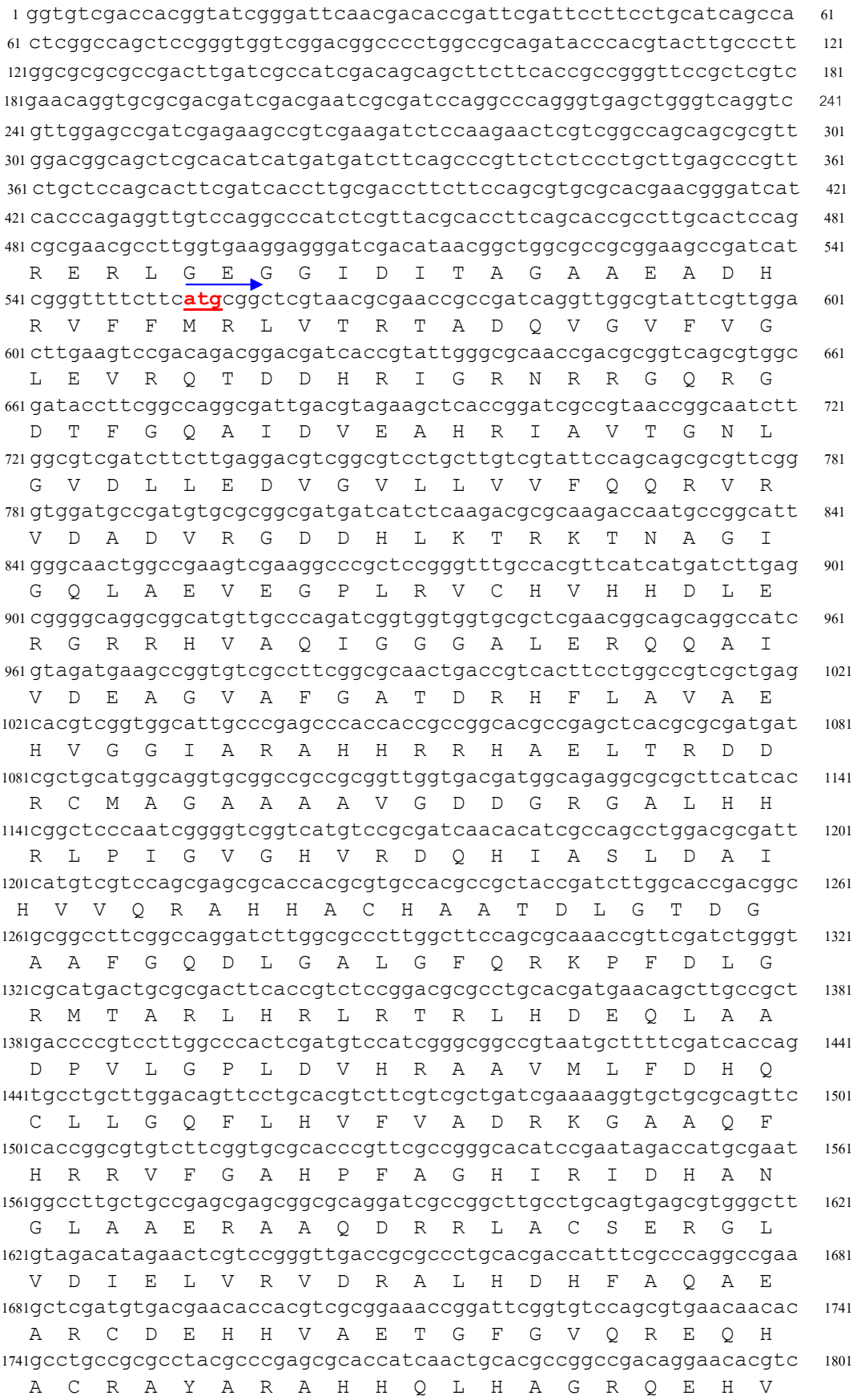
1 ggtgtcgaccacggtatcgggattcaacgacaccgattcgattccttcctgcatcagcca 61
R E R L G E G G I D I T A G A A E A D H
541 cgggttttcttcatggcggctcgtaacgcgaaccgccgatcaggttggcgtattcgttgga 601
R V F F M R L V T R T A D Q V G V F V G
601 cttgaagtccgacagacggacgatcaccgtattgggcgcaaccgacgcggtcagcgtggc 661
L E V R Q T D D H R I G R N R R G Q R G
661 gataccttcggccaggcgattgacgtagaagctcaccggatcgccgtaaccggcaatctt 721
D T F G Q A I D V E A H R I A V T G N L
721 ggcgtcgatcttcttgaggacgtcggcgtcctgcttgtcgtattccagcagcgcgttcgg 781
G V D L L E D V G V L L V V F Q Q R V R
781 gtggatgccgatgtgcgcggcgatgatcatctcaagacgcgcaagaccaatgccggcatt 841
V D A D V R G D D H L K T R K T N A G I
841 gggcaactggccgaagtcgaaggcccgctccgggtttgccacgttcatcatgatcttgag 901
G Q L A E V E G P L R V C H V H H D L E
901 cggggcaggcggcatgttgcccagatcggtggtggtgcgctcgaacggcagcaggccatc 961
R G R R H V A Q I G G G A L E R Q Q A I
961 gtagatgaagccggtgtcgccttcggcgcaactgaccgtcacttcctggccgtcgctgag 1021
V D E A G V A F G A T D R H F L A V A E
1021 cacgtcggtggcattgcccgagcccaccaccgccggcacgccgagctcacgcgcgatgat 1081
H V G G I A R A H H R R H A E L T R D D
1081 cgctgcatggcaggtgcggccgccgcggttggtgacgatggcagaggcgcgcttcatcac 1141
R C M A G A A A A V G D D G R G A L H H
1141 cggctcccaatcggggtcggtcatgtccgcgatcaacacatcgccagcctggacgcgatt 1201
R L P I G V G H V R D Q H I A S L D A I
1201 catgtcgtccagcgagcgcaccacgcgtgccacgccgctaccgatcttggcaccgacggc 1261
H V V Q R A H H A C H A A T D L G T D G
1261 gcggccttcggccaggatcttggcgcccttggcttccagcgcaaaccgttcgatctgggt 1321
A A F G Q D L G A L G F Q R K P F D L G
1321 cgcatgactgcgcgacttcaccgtctccggacgcgcctgcacgatgaacagcttgccgct 1381
R M T A R L H R L R T R L H D E Q L A A
1381 gaccccgtccttggcccactcgatgtccatcgggcggccgtaatgcttttcgatcaccag 1441
D P V L G P L D V H R A A V M L F D H Q
1441 tgcctgcttggacagttcctgcacgtcttcgtcgctgatcgaaaaggtgctgcgcagttc 1501
C L L G Q F L H V F V A D R K G A A Q F
1501 caccggcgtgtcttcggtgcgcacccgttcgccgggcacatccgaatagaccatgcgaat 1561
H R R V F G A H P F A G H I R I D H A N
1561 ggccttgctgccgagcgagcggcgcaggatcgccggcttgcctgcagtgagcgtgggctt 1621
G L A A E R A A Q D R R L A C S E R G L
1621 gtagacatagaactcgtccgggttgaccgcgccctgcacgaccatttcgcccaggccgaa 1681
V D I E L V R V D R A L H D H F A Q A E
1681 gctcgatgtgacgaacaccacgtcgcggaaaccggattcggtgtccagcgtgaacaacac 1741
A R C D E H H V A E T G F G V Q R E Q H
1741 gcctgccgcgcctacgcccgagcgcaccatcaactgcacgccggccgacaggaacacgtc 1801
A C R A Y A R A H H Q L H A G R Q E H V

Figure 9 Partial Nucleotide sequences of *ppsA* genes of *X. axonopodis pv. glycines* with 2,374 bb Deduced amino acid shown by single letter codes below. Potential ribosome-binding site (S/D) preceding one open reading frames (ORF) is underlined, horizontal arrows indicate orientation of transcription**.**

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7. Characterization of Nonpathogenic Mutant and Pathogenicity Related Function Analysis

The Tn5 flanking fragment had inserted in position site of Xag mutant strain KUMNTP2 of *ppsA* gene resulted in the failure of the pathogen to induce disease symptom on soybean and HR on tobacco. The experiment of *ppsA* gene has been characterized.

7.1 Biochemical characteristic test

The biochemical and physiological characteristics between wildtype mutant and complemented strains were not different. The wildtype and all mutant strains produced a yellow pigment and formed smooth colonies on NGA and green convex shaped of smooth margin on MXG medium. Other characteristics tested were presented in Table 10. They were aerobe gram negative, could produce catalase and amylase for starch degradation, gelatin liquefaction, levan formation and acid from glucose, sucrose, and dextrose as carbon utilization, and well grown in 5% salt medium. These results indicated the mutant was only affected in genes necessary for pathogenicity (Table 10).

7.2 Bacterial growth in *vitro*

To determine the effect of the mutation *ppsA* on bacterial growth in culture by incubating KUMNTP2 in synthetic medium containing sugar (glucose) and nonsugar (NaAc). The mean growth rate determined among Xag wildtype, mutant KUMNTP2, and complementation strains in M9 medium with glucose limitation (M9 supplemented with 0.5% glucose, M9 supplemented with 0.25% NaAC, and M9 alone) are presented in Fig.10. The study revealed that Xag wildtype and complementation strain were able grown in M9 supplemented with both glucose and NaAc as the sole carbon sources where the nonpathogenic mutant KUMNTP2, *ppsA* defective strain grew normally in only M9 supplemented with glucose, but it was unable grown in NaAC. The results indicate that a role of phosphoenal pyruvate for

growth of Xag on carbon sources requiring gluconeogenesis, growth of Xag mutant on minimal medium with 0.5 % glucose analyzed.

Our data compromised with previous work reported by Tang *et al.* (2005) in which *ppsA* mutant of *X. campestris* pv. *campestris* presented the same growth condition on glucose added medium. However, they do not demonstrate whether exogenous glucose is essential for recovering bacterial aggressiveness in inducing the disease severity on planthost, or the defective *ppsA* genes is required for HR induction on some nonhosts, and / or linked to some virulence factors secreted that we reported in this study.

Table 10 Physiological and biochemical characteristic of nonpathogenic mutant of *X. axonopodis* pv. *glycines* KUMNTP2 compared with wildtype and complemented strain**s**

Physiological and biochemical characteristic	Bacterial strains [1/]		
	Wildtype	Complemented	KUMNTP2
Colony morphology on NGA	Yellow, smooth circular	yellow , smooth circular	yellow , smooth circular
Gram stain reaction	negative	negative	negative
Catalase	+	+	+
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Levan formation	+	+	+
Salt tolerant (5%)	+	+	+
Salt tolerant (7%)	-	-	-
Carbon Utilization			
Sucrose	+	+	+
Glucose	+	+	+
Dextrose	+	+	+
Colonies growth on MXG	green convex shaped	green convex shaped	green convex shaped

[1/] + = positive reaction , - = negative reaction. ; Teste

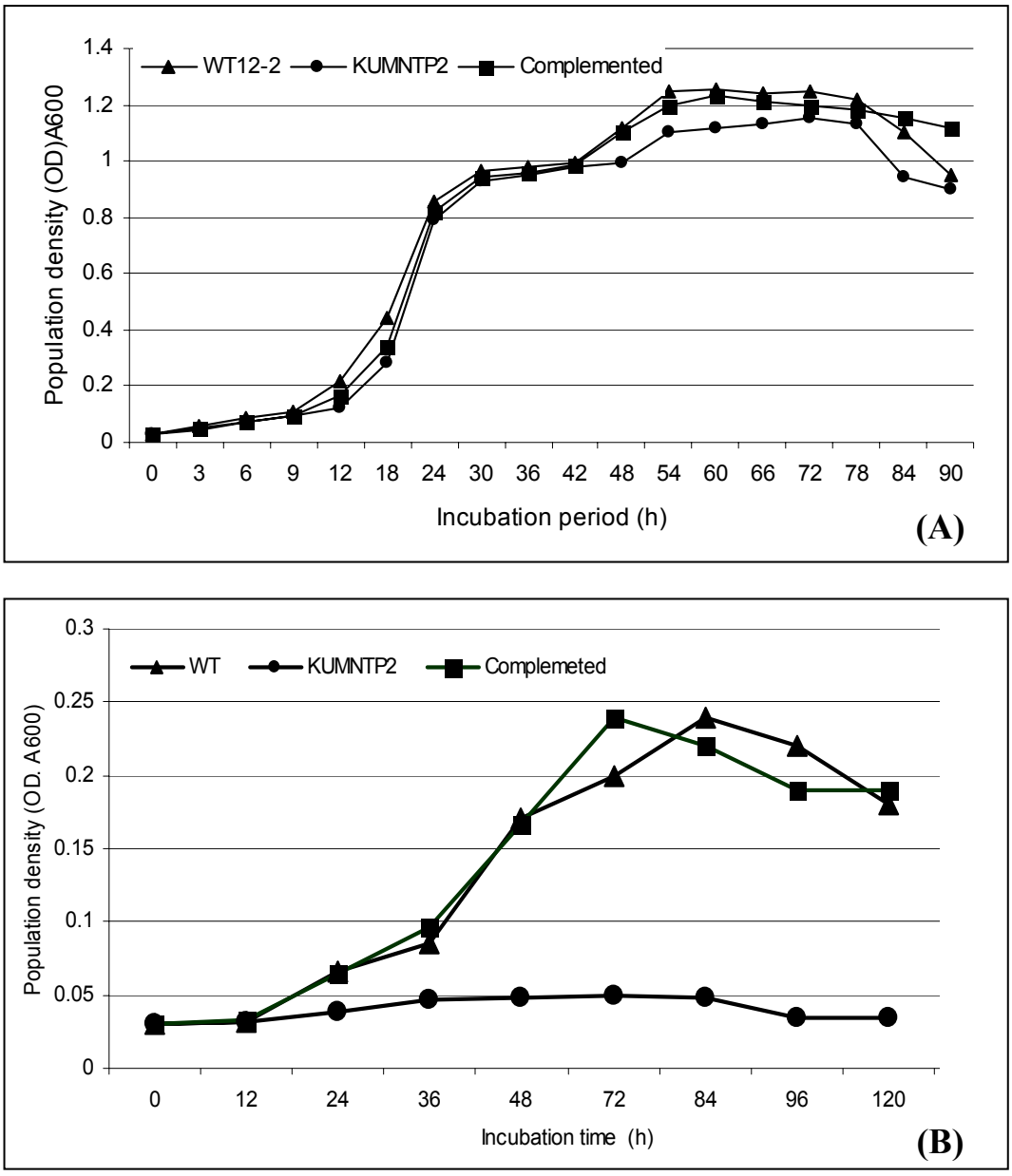

Figure 10 Growth rate of *X. axonopodis* pv. *glycines*, wildtype (Wt) and mutant (KUMNTP2) comparing with complementation (Cp) in minimal medium (M9) containing with 0.5 % glucose (A) and 0.25 % sodium acetate (NaAC) (B).

7.3 Growth of mutant in planta and symptom expression

To investigate whether the virulence of the pathogen Xag is affected by a mutation in *ppsA*, the virulence of mutant strain KUMNTP2 was tested by infiltrated inoculating onto the leaves of host plant susceptible soybean cultivar (SJ4) grown in a greenhouse condition as materials and methods described. In *planta* experiment, multiplication level of mutant KUMNTP2 was significantly lower than wildtype and complementation strains in all tested periods from 0 to 120 h after inoculation (Table 11 and Fig.11). Population density of wildtype and complementation strains showed rapid multiplication from the initiate inoculation source of 1x108 to 5.2 $x10^8$ and 2.5 $x10^9$ cfu/ml at 18 and 120 h post inoculation respectively. The mutant KUMNTP2 showed lower level of population density at all incubation periods. The multiplication of mutant was maintained at the range of $1x10^8$ to 2.7 x 10^8 cfu/ml at 0 to18 h, and slowly increased at $5.1x10^8$, $5.5x10^8$, $6.7x10^8$, and $7.4x10^8$ cfu/ml at 48, 72, 96, and 120 h post inoculation (Fig.11). The total population of the *ppsA* mutant increased slightly during the first 24 h post inoculation and remained lower than the wild type throughout the experiment.

These data suggest that *ppsA* is essential for the early stage of pathogen multiplication in soybean plant and critical for persistence in the later stage of infections. Also, the results indicate that an intact gluconeogenic pathway is required for full virulence and the reduced virulence is probably related to the reduced bacterial numbers of the mutant in plant tissues. The reduction growth of mutant strain in host plant was affected by the several factors including environmental aspects, plant physiology and development, and the expression of pathogenicity gene and virulent factors by the bacterial cells (Sigee, 1993). The results could indicate that population density of bacterial pathogen is necessary for their pathogenesis and direct or indirect effect to infection process. As the results described, the multiplication of Xag mutant KUMNTP2 was deficient and may be related to the development of epiphytic fitness to pathogenicity phase.

Table 11 Multiplication of *X. axonopodis* pv. *glycines* mutant KUMNTP2, wildtype, and complemented strains in the infiltrated soybean leaf tissue at different incubation period

Incubation period (h)	Population density (cfu/ml/leaf disc)[1/]		
	Bacterial strains		
	KUMNTP2	Wildtype	Complement
0	$1x10^8$	$1x10^8$	$1x10^8$
6	$2x10^8$	$2.5x10^8$	$2.4x10^8$
12	$2.3x10^8$	$3x10^8$	$2.8\ x10^8$
18	$2.7x10^8$	$5.2x10^8$	$5.3\ x10^8$
24	$3.8x10^8$	$1.2\ x10^9$	$1.1\ x10^9$
36	$4.3x10^8$	$1.5\ x10^9$	$1.5\ x10^9$
48	$5.1x10^8$	$1.7\ x10^9$	$1.7\ x10^9$
72	$5.5x10^8$	$1.9\ x10^9$	$1.9\ x10^9$
96	$6.7x10^8$	$2.1\ x10^9$	$2\ x10^9$
120	$7.4x10^8$	$2.52\ x10^9$	$2.5\ x10^9$

[1/] Leaf discs of 5 mm diameter of soybean were cut from the center of the infiltrated zone at the intervals from 0-120 h post infiltration (four discs for each treatment and each time interval were average). Samples were placed individually in 1 ml SDW, triturated and dilutions at 10^{-7} to 10^{-12} were plated on LB agar medium supplemented with kanamycin (50 µg/ml), refampicin (25 µg/ml) and tetracyclin (25 µg/ml) for detected Xag mutant and wildtype and complemented strains respectively. The population density of bacterial strains couted from each tesed plated were calculated and adjusted by colony forming unit per ml per leaf disc (cfu/ml).

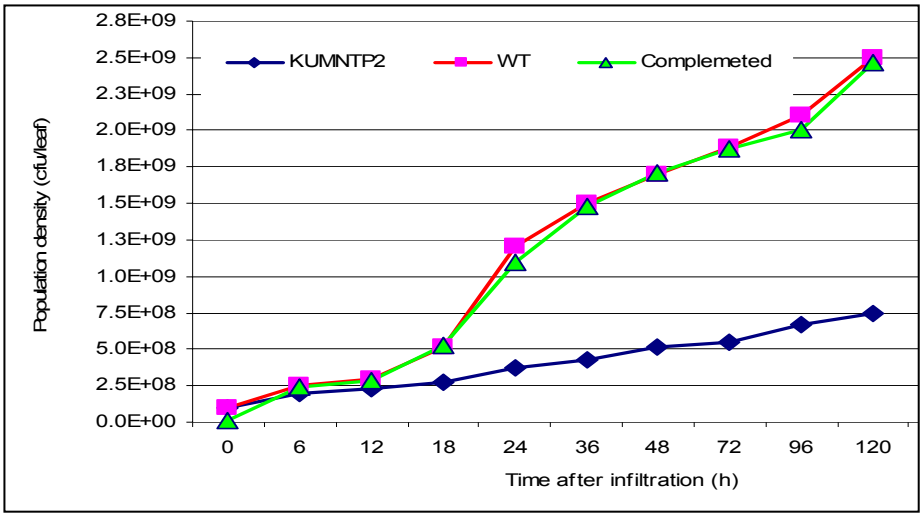

Figure 11 Population density of *X. axonopodis* pv. *glycines* wild type (WT), mutant KUMNTP2 and complementation strains detected from infected leaves of soybean at various incubation periods.

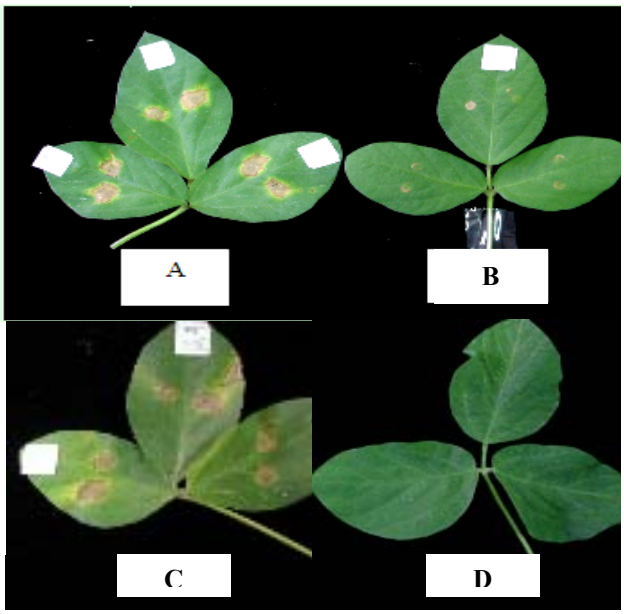

Figure 12 Disease symptoms on soybean leaves at 120 h post inoculated with each bacterial suspension were demonstrated from (A) to (D). Wildtype and complement showed clearly aggressive symptom (A and C), whereas mutant KUMNTP2 could not induce disease on tested plants (B), including negative control infiltrated with distilled water (D).

To know whether the different levels of cell density in the lesion leaves is related to the ability of mutant to induce disease symptom on susceptible soybean cultivar, wildtype and mutant strains was infiltrated inoculation with varied the initial concentration of cell suspensions. The result showed that the mutant strain KUMNTP2 could induce neither any disease symptoms nor necrotic lesions in the area of infiltration site at 10^8 cfu/ml concentration, where wildtype and complementation strains showed clearly necrotic lesions around infiltration area within 72 h (the initial symptom could be detected at 48 h) (Table 12). The initiate symptoms caused by wildtype and complementation strains have shown with the water soaking spot and developed to dried and brown colors surrounded with halo yellow. The size of lesions of infiltrated inoculation was also increased as the incubation period increased (Table 12 and Fig. 12).

For foliar spray inoculation assay, soybean plants inoculated with wildtype and complemented strains showed disease incidence within 4 days after inoculation with disease severity of 25 and 12% of infected leaf area respectively (Table13). The disease induced by wildtype showed highest severity with 57 and 78 % at 7 and 10 days after inculcation respectively. This results related with the population density of bacteria and their multiplication in host plant as the population density of wildtype and complemented strains fast increased from the initiate inoculation source where as mutant strain KUMNTP2 showed the lower level of population density at all incubation periods. The accumulation and multiplications of wild type strain could induced disease symptom at 72 h after inoculation at the population density of highest $1.9x10^9$ cfu/leaf (Table11). In contrast, the population density of mutant strain showed slowly increased from 6 h after infiltration and could not induced disease symptom on soybean leaves as affected with the infection process.

Table 12 Efficacy of *X. axonopodis* pv. *glycines* mutant, wildtype, and complemented strains for induced bacterial pustule symptom on susceptible soybean SJ4 cultivar by individual bacterial inoculation by tissue infiltration and foliar spray with cell concentration of 0.2 OD (A_{600nm})

Time after inoculation (h)	Disease incidence [1/] / Disease severity (%) [2/]					
	Bacterial strain[3/]					
	Tissue infiltration			Foliar spray		
	WT	MT	CP	Wt	Mt	CP
0	-	-	-	-	0	0
12	-	-	-	-	0	0
24	-	-	-	-	0	0
36	-	-	-	-	0	0
48	+	-	-	-	0	0
72	++	-	+	-	0	0
96	+++	-	+	10	0	0
4 days	++++	-	++	25	0	12
7 days	++++	-	++	57	0	36
10 days	++++	+	+++	78	0	65

[1/] Disease incidence subjected to infiltration method monitored for development of tissue collapse and necrosis that + = slightly necrotic spots visible in infiltrated area ; ++ = moderated necrosis at margins of infiltrated area ; +++ = clear necrosis of the entire infiltrated area with brown tissue; ++++ = completely dried at infiltrated area; and - = no visible spot was observed .

[2/] Diseases severity subjected to foliar spray method expressed as percent leaf area infection as described by Prathuangwong *et al.* (1993).

[3/] Bacterial strains including wildtype (WT), mutant (MT) and complement (CP).

7.4 Effect cell density on disease induction

The potential of population density for induced disease symptom of Xag mutant strain were tested with varied cell concentration at 0.2, 0.5, and 1.0 OD and inoculated host plant with infiltration assay comparing with wild type and complement strains. The result revealed that all Xag strains could induce necrotic lesions which different typical symptoms on tested plants depended on cell concentration and incubation period (Table 13). It was apparent that the Xag wild type and complement strains could induced necrotic lesion within 48 h with all cell concentration of 0.2, 0.5 and 1.0 OD (A_{600nm}) whereas mutant strain could not induce any symptom when infiltrated host plant with cell concentration at 0.2 OD (A_{600nm}) for 48 h. The first lesion induced by mutant could be observed at 72 h post infiltration at the cell concentration of 0.5 and 1.0 OD (A_{600nm}) (Table 13 and Fig. 13). The size of necrotic lesions showed different patterns depend on incubation period and number of pathogen. The disease symptom induced by wild type and complemented strains were clearly expressed at 72 h post infiltration at concentration of 0.5 and 1.0 (A_{600nm}), respectively where as mutant strain showed at 96 h post infiltration at concentration of 1.0 OD (Table 13). These results indicated that disease induction on tested plant affected relating cell concentration and incubation period governed by virulent genes with induced *ppsA* gene.

Multiplication of bacterial pathogen to induce disease are involved in several factor as mention by Sigee (1993). Plant infection and disease development depended on a number of factors including the particular host-pathogen interaction, critical environmental conditions, physiological stress of the host plant, and the attachment of minimal thresholds of the pathogen (Malvick and Moore 1988). However, the phytopathogenic bacterial Xanthomonads are not capable of progressively colonizing plant surface. Especially on leaves, the ephiphytic population of xanthomonads fluctuate widely and the limiting factors is aviability of free water (Leben and daft, 1967). These information can be suggested that the loss of pathogenicity of mutant strain was related with their capability to maintain or multiply in host tissue

Table 13 Potential of *X. axonopodis* pv. *glycines* mutant, wildtype, and complement strains induced bacterial pustule symptom on susceptible soybean SJ4 cultivar by tissue infiltration with various cell concentrations

Incubation period (h)	Cell-concentration (cfu/ml)	Disease incidence [a/]		
		Bacterial strain		
		Wild type	KUMNTP2	Complement
48	10^8	-/+	-	-
	10^9	+	-	-/+
	10^{11}	+	-	+
72	10^8	+	-	+
	10^9	++	+	+
	10^{11}	+++	+	++
96	10^8	++	-	+
	10^9	+++	+	++
	10^{11}	+++	++	++
108	10^8	++	-	++
	10^9	+++	++	++
	10^{11}	+++	++	++
120	10^8	++	-	+++
	10^9	+++	++	++
	10^{11}	++++	++	+++

[1/] Infiltrated areas were monitored for development of tissue collapse and necrosis from 48 to 120 h post inoculation in which + = slightly necrotic spots visible in infiltrated area ; ++ = moderated necrosis at margins of infiltrated area ; +++ = clear necrosis of the entire infiltrated area with brown tissue ; ++++ = completely dried at infiltrated area ; and - = no visible spot was observed.

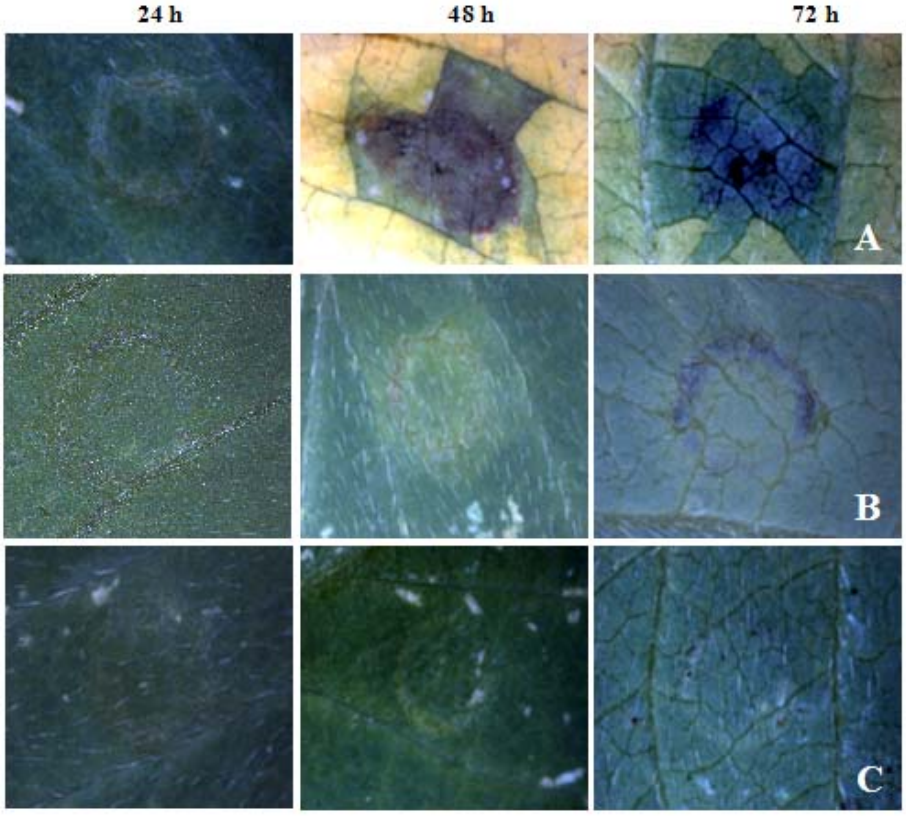

Figure 13 Necrotic lesion developed on soybean leaves observed under stereo microscope (4000X) at 24, 48, and 72 h after infiltrated with cell suspension of *X. axonopodis pv. glycines* wildtype (A) mutant strain (B) and negative control inoculated with dH_2O (C). Wildtype strains could induce necrotic lesion with 48 h with concentration of 10^8 cfu/ml whereas mutant strain could not induce any symptom.

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7.5 Effects of glucose on pathogenicity

The *ppsA* in this study revealed to be involved in correlation between glucose and the pathogenesis of Xag. Thus we analyzed whether there are interactions between the expression of *ppsA* and that of pathogenicity genes in plant under limited glucose condition. The potential for disease induction and virulence of Xag mutant was studied comparing with wildtype and complementation strains by mixed inoculated with 10% (v/v) glucose by infiltration and spray inoculation. The results of infiltration assay showed that the mutant KUMNTP2 recovered disease induction with a small necrotic lesions in the infiltrated area at 3 days after glucose mixture, where as wildtype and complementation strains showed initiate necrotic lesions around infiltration area within 36 h and the size of lesions was increased as the incubation periods increased from 2 to 5 days observed (Table 14).

In the foliar spray inoculation, soybean plant inoculated with wildtype and complementation strains showed disease incidence within 4 days after inoculation with disease severity of 25 and 12% (Table14). The disease severity induced by wildtype showed highest severity with 44, 62, and 84% at 5, 7, and 10 days after inoculation respectively. While mutant strain exhibited the pathogenicity recovered with disease incidence at 7 and 10 days after cell suspension mixed with 10% glucose at the level of disease severity of 14 and 24 % respectively. The *ppsA* mutant seemed to produce some secreted protein effectors when applied with exogenous glucose, which suggests that mutant do not produce enough glucose to result in severe disease expression. The mutant had an effect on the growth rate in planta that showed loss of virulence associated with mutation of *ppsA*. The mutation was then, has detectable effect on bacterial infection with plant.

Table 14 Efficacy of *X. axonopodis* pv. *glycines* mutant, wildtype, and complement strains on induced bacterial pustule symptom on susceptible soybean SJ4 cultivar by foliar spray inoculation with and without 10% glucose (v/v)

Time after inoculation day / h	Disease incidence [1/] / Disease severity (%) [2/]											
	Application treatment											
	Inoculation without glucose [3/]						Inoculation with glucose [3/]					
	Infiltration			Foliar spray			Infiltration			Foliar spray		
	WT	MT	CP	WT	MT	CP	WT	MT	CP	WT	MT	CP
2 / 48 h	-	-	-	-	0	0	++	-	+	-	0	0
3 / 72 h	+	-	-	-	0	0	++	+	+	-	0	0
4 / 96 h	++	-	+	10	0	0	+++	+	+	25	0	12
5 / 120 h	++	-	++	25	0	12	++++	+	++	44	0	23
7 / 168 h	+++	-	++	57	0	36	++++	++	++	62	14	38
10 / 240 h	++++	+	+++	78	0	65	++++	++	+++	84	24	67

[1/] Disease incidence subjected to infiltration method monitored for development of tissue collapse and necrosis that + = slightly necrotic spots visible in infiltrated area ; ++ = moderated necrosis at margins of infiltrated area ; +++ = clear necrosis of the entire infiltrated area with brown tissue; ++++ = completely dried at infiltrated area ; and - = no visible spot was observed .

[2/] Diseases severity subjected to foliar spray method expressed as percent leaf area infection as described by Prathuangwong *et al.* (1993).

[3/] Bacterial strains including wild type (WT), mutant (MT) and complement (CP).

8. Extracellular Enzyme Secretory, EPS Production, and Interaction of Disease Severity

Enzyme plate assay for detection exoenzymes secreted by Xag strains revealed that wildtype and complementation strains were clearly secreted cellulase in tested plates (Fig. 14) where as the mutant strain have decreased level of cellulase production that had no clear zone observed in tested medium of cellulase indicator. In the secretion of alpha amylase, the result showed that all wild type, mutant and complementation strains could produce equal volume to their enzymes, but could not detect the protease. Mutation of the *ppsA* then, had no effect on the level of alpha amylase production of Xag and this medium, starch medium remains a good choice for investigation working on protein secretion of type II. These results indicate that cellulase secretion requires induction from *ppsA*. Cellulase secretion as one of type II protein secretion systems may be dependent on *ppsA* homolog that many Gram-negative plant pathogenic bacteria employ type II to deliver effector proteins differently into the host cell during infection with consistent to the report that several secreted proteins including auxin, cytokinin, toxin, EPS, protease, and celluase are the virulent factors of Xag (Fett and Dunn, 1987).

The activity of cellulase was also assayed quantitatively in extracellular, periplasmic and cytoplasmic fractions. Most of the cellulase activity were detected in the periplasmic fraction and very few in the extracellular fraction of mutant strain (Fig. 15). Wildtype showed high activity of its extracellular expression, whereas periplasmic and cytoplasmic fractions had very little activity. Cellulase activity was restored in the complement clones indicating that the secretion of cellulase to the extracellular space was interrupted by the mutation with insertion sites in the *ppsA* genes that might be indispensable to the normal function of the cellulase secretion. The data also suggest that cellulase is secreted by the type II protein secretion system in Xag and that mutant in the *ppsA* genes affect its secretion, resulting in its accumulation in the periplasm and cytoplasm. In generally, protein secretion for the extracellular environments of Gram-negative bacteria have to cross two membranes during their journey across the bacterial cell envelope. This involves translocation

across the cytoplasmic membrane and the outer membrane, which are separated by the periplasmic compartment and the peptidoglycan layer (Pohlschr *et al.,* 1997).

To determine whether the reduced virulence of Xag mutant was affected from its deficient cellulase production, the efficacy of cellulase for enhanced disease severity was defined by conducting phytotoxic activity tested. In soybean plant experiment, the results shown the typical symptom as necrotic lesions around the infiltration site on tested soybean leaves were different exhibited. The necrotic lesions were variable with color and size of symptoms depended on concentration and incubation period. Among concentration of 100 % cellulase, and diluted concentration of 1:1 and 1:2 however, they showed aggressiveness to induce cell necrotic within the same period of 3 days (Table 16). The initiate symptom observed have shown with the small yellow lesion area around the infiltrated site, the symptom was expanded to adjacent area with brown color occurred in the middle of lesions surrounded with halo yellow. The size of lesions was increased as the incubation periods increased. In lower concentrations, initiate lesions showed slowly. This result could be confirmed that cellulase played a virulent factor for Xag to enhance disease severity an full virulence.

Maximum levels of EPS production by the three strains were detected in shaked culture media with the method described (Poplawsky and Chun, 1997). The 72-h cell-free culture of Xag mutant was lower contained EPS production comparing with wildtype and complementation strains. Wildtype showed a higher amount of EPS production of 32.0 ug/ml followed by complementation strain with a weight of 30.0 µg/ml (data not show). The amount of EPS produced by the *ppsA* mutant was six fold lower than that of wildtype cells that it could be detected at 5 ug/ml. Culture supernatants of *ppsA* mutant restored EPS production in the complementation strain to the level seen in wildtype. This suggests that reduction of EPS can compensate for absence of some signalling protein in EPS synthesis and supports the idea that *ppsA* affects the production of EPS via the lower cell multiplication and cell-cell communication .

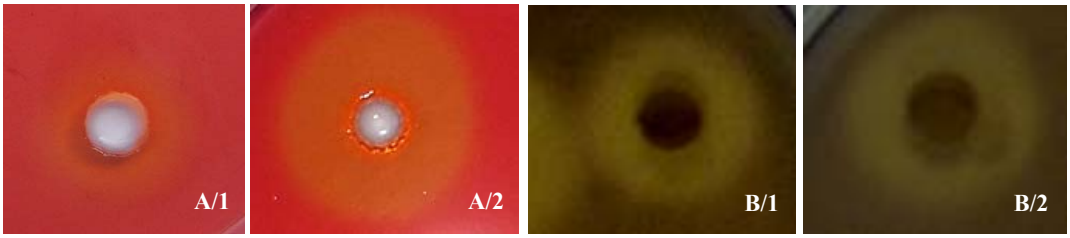

Figure 14 Extracellular enzyme plate assay revealed the nonpathogenic mutant strain KUMNTP2 (A1) was deficient produced cellulase compared with wildtype (A2). Its was not different on alpha amylase production among KUMNTP2 (B1) and wild type (B2).

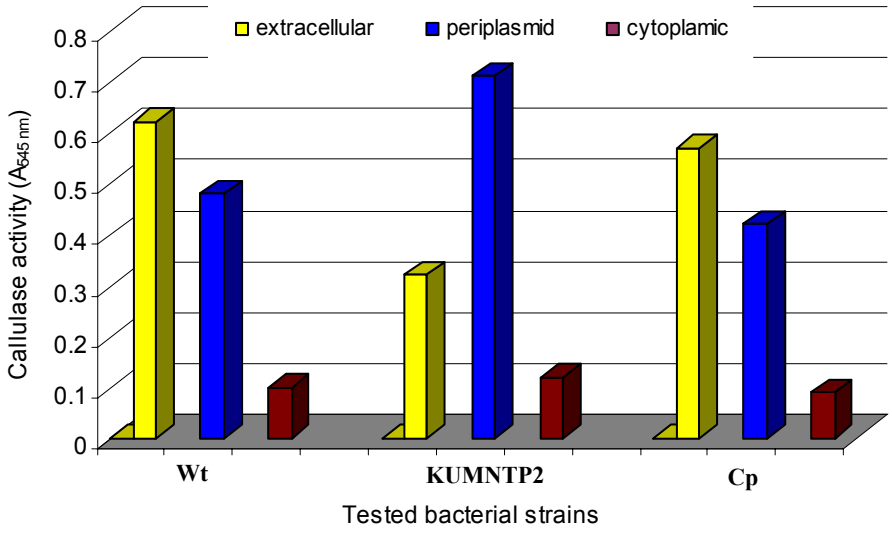

Figure 15 Distribution of cellulase activity in various cellular fractions, extracellar, periplasmic, and cytoplasmic membrane of *X. axonopodis* pv. *glycines* wildtype No.12-2 (Wt), mutant (KUMNTP2) and complementation (Cp) strains.

Table 15 Quantification of cellulase to induce necrotic lesions on soybean leaves tested by single infiltration with various cellulase concentrations

Dilution of cellulase : H_20	Development of necrotic lesion (DAI)[1/]					
	1	2	3	4	5	6
1:1	-	-	+	++	++	+++
1:2	-	-	+	+	++	+++
1:3	-	-	+/-	+	++	++
1:4	-	-	-	+/-	+	++
1:5	-	-	-	+	+	+
100% Cellulase	-	+	++	++	+++	++++
dH20	-	-	-	-	-	-

[1/] Infiltrated areas were monitored for development of tissue collapse and necrosis from 1-6 days post inoculation in which + = slightly necrotic spot visible in infiltrated area ; ++ = moderated necrosis at margins of infiltrated area ; +++ = clear necrosis of the entire infiltrated area with brown tissue ; ++++ = completely dried at infiltrated area ; and - = no visible spot was observed ; and DAI = day after infiltration.

9. Effect of Bacterial Density on HR Induction

The activity of HR induction was related between bacterium cell and host plant. To determine the effect of cell-concentration of Xag wildtype, mutant, and complementation strains on HR induction was investigated on tobacco and tomato leaves as methods described. The results revealed that Xag wildtype and complementation strains could induce HR lesions on tomato and tobacco leaves with all of the cell concentrations tested. The minimum concentration of Xag wildtype and complementation strains that consist entry caused complete collapse of infiltration tissues after 24 h was about $2x10^8$ cfu/ml (Table 16). For the mutant KUMNTP2, it was failed to induce visible HR on tobacco but still induced cell death on tomato. The HR did not develop when a cell concentration of mutant at $2x10^8$ cfu/ml was injected, and the mild chlorotic spot was occurred within the infiltrated zone when the cell concentration was increased to $5x10^9$ cfu/ml. However, the clear HR induction was restored when the cell concentration of mutant was increased to $2x10^{10}$ cfu/ml with completed tissue collapse and dryness within 24 h after infiltration (Table 16). The results indicate that *ppsA* might be not affected to induce the same signalling pathways between these nonhost plants, tobacco and tomato. Number of cell concentration and type of tested plants are the one factor for HR induction. Bacterial cells sense their population density through a cell-cell communication system with production of signalling and receptors molecule and trigger expression of particular genes when the density reaches a threshold including exoenzymes, EPS, and *hrp* genes. The interfere of *ppsA* was related to carbon source utilization and cell multiplication of Xag on host plants and might be direct or indirect effect to quorum sensing or biofilm formation (Poplawsky and Chun, 1997 ; Poplawsky *et al.,* 1998). These results suggest that *ppsA* disrupted was also effected to the quorum sensing of Xag which an important roles in plant-bacterial interaction.

Table 16 Ability of various cell concentrations of *X. axonopodis* pv. *glycines* to cause hypersensitive response on tobacco and tomato nonhost leaves

X. axonopodis pv. *glycines* strain	Concentration (cfu/ml)	HR induction [1/]	
		Tobacco	Tomato
Wildtype No. 12-2	$2x10^8$	+	+
	$5x10^9$	+	+
	$2x10^{11}$	+	+
Mutant KUMNTP2	$2x10^8$	-	+
	$5x10^9$	-	+
	$2x10^{11}$	+	+
Complementation strain	$2x10^8$	+	+
	$5x10^9$	+	+
	$2x10^{11}$	+	+

[1/] HR= hypersensitive response ; + = positive reaction, - = negative reaction, infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h post inoculation. The negative reaction is no visible nercrosis. The positive reaction is complete collapse and necrosis of the entire infiltrated area.

10. Defense-related enzyme induced by Xag mutant

The results obtained in this study revealed that treatment of soybean plants with either separated- or co-inoculation induced the expression of SAR-related plant genes with accumulating 4-defense related chemicals detection. However, the coinoculation of mutant pretreated plants was induced and accumulated significantly in greater amount of β-1,3 glucanase and phenylalanine ammonia lyases (PAL). When each enzyme was considerate, the highest level of β-1,3-glucanase accumulation was detected in soybean leaves treated with mutant and challenged with wildtype, followed by treatment inoculated with mutant and challenged with complement, and inoculation with mutant alone respectively (Fig.3). Accumulation of β-1,3-glucanase obtained from mutant inducer started one day after challenge inoculation with pathogenic wildtype. The maximum accumulation observed on the 3rd day after challenge inoculation. Soybean plants inoculated with the pathogen alone also recorded increased accumulation of β-1,3-glucanase, but accumulation started on 2nd day after the pathogen inoculation and drastically declined at 4 days after inoculation. The maximized β-1,3-glucanase accumulation at the 3rd day after challenge inoculation revealed its activity levels induced by mutant was 0.5 and 0.6 folds, higher than wildtype and complement respectively. Moreover, the accumulation of β-1,3-glucanase was less compared to mutant challenged with the pathogen. There was no marked change in plants treated with mutant alone during the time course of experiment period and the accumulation of this enzyme remained higher compared to the untreated control (Fig.3).

Similar results detected that the higher activity of PAL was observed in soybean leaf tissues inoculated with Xag mutant alone and coinoculated with mutant-wild type and coinoculated mutant-complemented strain treatments respectively. The activity of PAL on soybean leaves inoculated with mutant was higher than in the leaves inoculated with wildtype and complement strains alone. PAL activity detected was accumulated and increased from 2nd to 5th day and showed highest accumulation level at 4th day after Xag inoculation with activity level of 23.6, 25.3, 23.3, 27.6, and 26.4 nmol trans-cinnamic acid min^{-1} mg^{-1} protein for treatments of individual

inoculated with wildtype, mutant, complemented strain and coinoculated with mutant-wild type and mutant-complemented strains respectively, whereas treatment inoculated with SDW showed the less of PAL accumulation at 18 nmol trans-cinnamic acid min^{-1} mg^{-1} protein (Fig.3).

Phenolic compounds and POX detected in this study were not significant in activity increased in all treatments investigated. The accumulation of phenolics was increased within one day after inoculation and reached maximum at the 3rd day with phenol activity levels of 173.30, 174.63, 168.43, 173.58, and 172.96 µg catechol mg^{-1} protein for treatments of individual inoculated with wildtype, mutant, complement strains and coinoculated with mutant and wildtype challenged; and mutant and complement strains challenged respectively. SDW showed phenolics compounds was accumulated lower activity level at 118.18 µg catechol mg^{-1} protein (Fig.2). In the POX activity assay, its maximum activity was also observed at 3rd day after bacterial inoculation and showed no difference level when inoculation with Xag wildtype, mutant, and complement alone and coinoculated with mutant and either wildtype or complement with enzyme activity levels at 4.30, 4.28, 4.16, 4.31, and 4.36 min^{-1} mg^{-1} protein respectively. However, treatment inoculated with SDW as negative control was also stimulated POX enzymes, but the accumulation level was significant less than that of those Xag strains with 3.67 at 3 days after inoculation (Fig. 2).

The accumulation of these two enzymes, β-1,3 glucanase and PAL, was related with the severity of disease induction on tested plants. Soybean plants inoculated with wildtype and complement strains showed disease incidence within 7 days after inculcation with highest disease severity of 59.7 and 39.7% leaf area infiltrated respectively whereas mutant strain showed a slightly disease severity with 0.6 % (Table 3). Coinoculation treatment of mutant-wildtype and mutant-complemented strains showed lower disease severity compared to individual inoculated with wildtype and complemented strain alone at disease severity of 13.45 and 14.23 % respectively. In this investigation, KUMNTP2 mutant was clearly found to reduce the incidence of bacterial pustule disease in soybean plants and indicated that the activity and accumulation of defense related-enzymes, β-1,3 glucanase and PAL in soybean leaf

tissues have been induced by KUMNTP2 in response to challenge inoculation with pathogenic Xag wildtype. They are key enzymes in the production of PR protein and phytoalexin that inhibited bacterial invasion in plants.

Table 17 Severity of bacterial pustule disease at 7 days after single- and co-inoculation of *X. axonopodis* pv. *glycines* (Xag), wildtype, mutant KUMNTP2, and complement strains

Treatment[1/]	Disease severity[2/] (%)
Xag wild type	59.7 d
Xag mutant KUMNTP2	0.6 a
Xag complement	39.7 c
Xag mutant and wild type	13.5 b
Xag mutant and complement	14.2 b
ddH_2O	0 e
CV.	6.7

[1/] Foliar spray was inoculated onto soybean plants using hand-hold spray bottles (Prathuangwong, 1984).

[2/] Diseases severity was expressed as percent leaf area infection with *X. axopodis* pv. *glycines* evaluated by the method of Prathuangwong *et al.* (1993) at 7 days after inoculation. Means in the column followed by the same letter are not significantly different ($P< 0.05$) according to Duncan's New Multiple Rang Test.

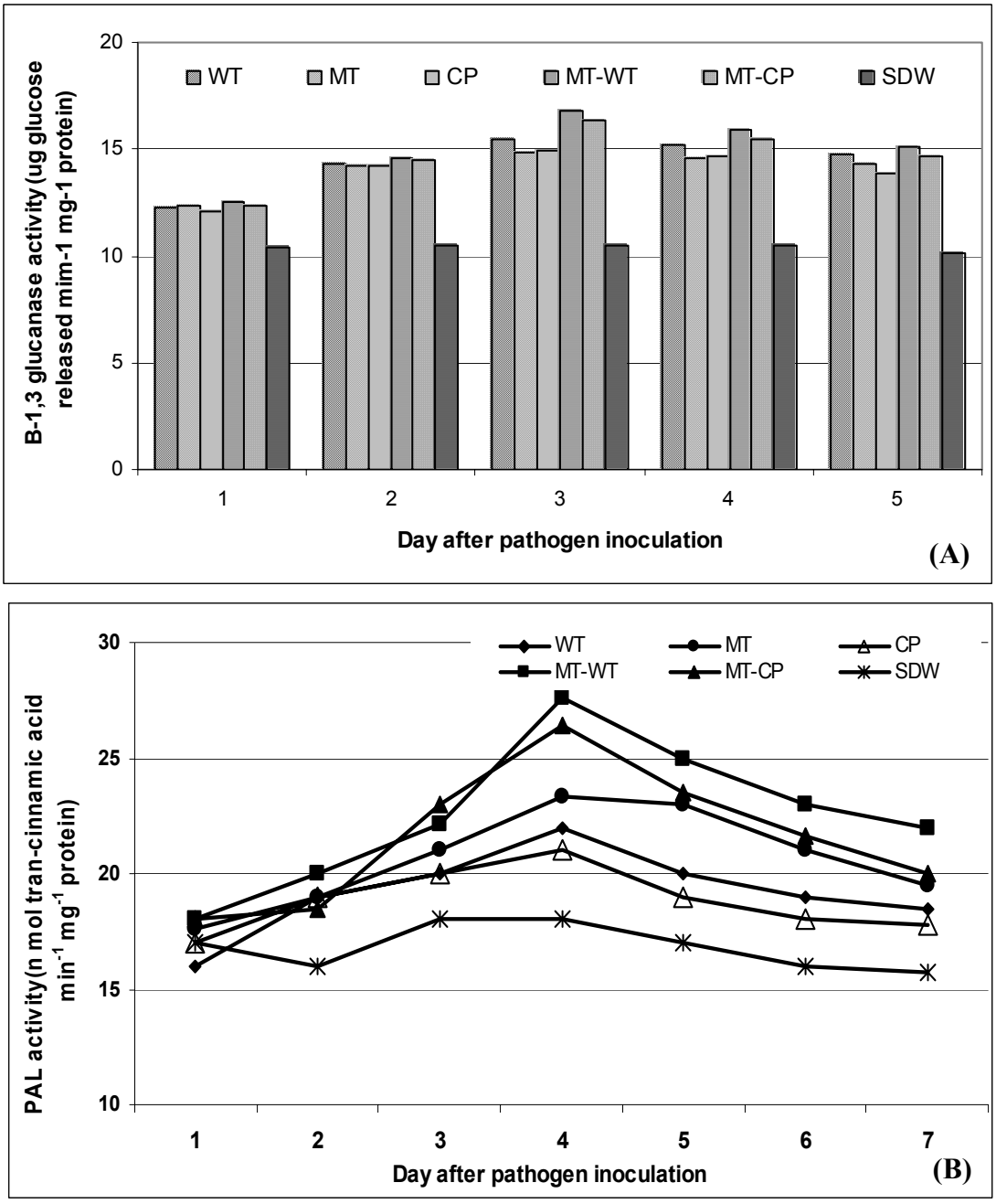

Figure 16 Accumulation of β -1,3-glucanase (A), and PAL activity (B) on susceptible soybean SJ4 leaves after inoculation with *X. axonopodis* pv. *glycines* wild type (WT), mutant KUMNTP2 (MT), and complement (CP) strains compared with co-inoculated of mutant-wildtype (MT- WT) and mutant–complement (MT-CP) and sterile distilled water (SDW) at various incubation times.

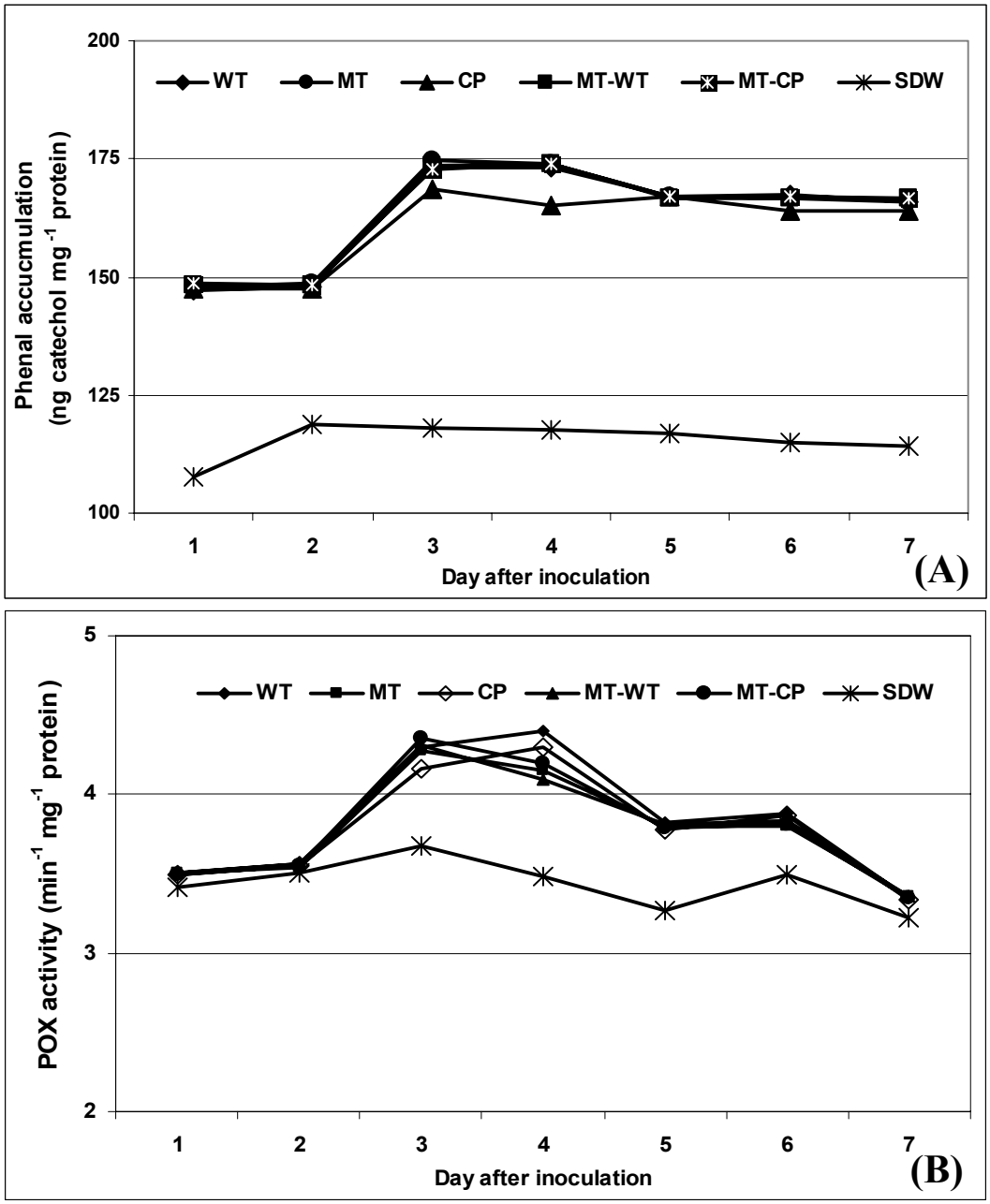

Figure 17 Accumulation of phenolics (A), POX activity (B), on susceptible soybean SJ4 leaves after inoculation with *X. axonopodis* pv. *glycines* wildtype (WT), mutant KUMNTP2 (MT), and complement (CP) strains compared with co- inoculated of mutant-wildtype (MT- WT) and mutant–complement (MT-CP) and sterile distilled water (SDW) at various incubation times.

11. Bacterial growth in *planta* and disease reduction

Compatibility of bacterial growth was measured to investigate the effects of related-enzymes mediated defense responses on the virulent of pathogenic wildtype by tissue infiltration at 24 h after foliar sprayed inoculation with mutant KUMNTP2. The growth of mutant, complement and wildtype was measured to compare how these three strains responded to related-enzymes mediated defense in soybean host plants. The relatively growth rate of each Xag strain was observed at 24, 48, 72, and 96 h after inoculation at infiltrated areas. Treatments infiltrated by wildtype with- and without- pre-foliar sprayed mutant showed different multiplication rates on tested soybean leaves. Population density of wildtype was significant greater in soybean leaf tissues when inoculated alone which the population density of $3x10^7$, $5x10^8$, $3x10^{10}$, $5x10^{11}$ cfu/ml at 24, 48, 72, and 96 h after infiltration respectively, whereas the population of wildtype was lower and delayed multiplied when inoculated at 24 h post-foliar sprayed with mutant strain at population density of $1.9x10^5$, $9.5x10^7$, $2.8x10^8$, and $6.2x10^8$ cfu/ml of 24, 48, 72, and 96 h after infiltration respectively (Fig. 4). Population density of mutant in infected soybean leaves showed lowest growth at all incubation periods tested. While the inoculation between mutant and complement strains showed density of bacterial cell in plant tissues as same treatment as inoculated wildtype. This result could be confirmed that pre-inoculation with Xag mutant was related to induction some defense responses suppressing growth rate of wildtype in host tissues since the invidence of bacterial inhibition was not observed when the two-related strains, mutant and wildtype were plated co-culture tested (data not shown).

Table 18 Multiplication of *X. axonopodis* pv. *glycines* strains after individual and co-infiltration into mesophyll layer of susceptible soybean cultivars SJ4 at various incubation period.

Inoculation treatment[a/]	Population density (cfu/ml)			
	Post inoculation (h)			
	24	48	72	96
Wild type (Wt) alone	$3x10^7$	5×10^8	3×10^{10}	5×10^{11}
Mutant (Mt) alone	$4x10^3$	5×10^4	2×10^5	5×10^5
Complement (Cp) alone	$2x10^7$	4×10^8	3×10^{10}	5×10^{11}
MT and WT	4×10^5	2×10^6	$7x 10^6$	8×10^7
MT + Cp	2×10^5	8×10^5	3×10^6	5×10^7
DH_2O	-	-	-	-

[1/] Bacterial strains including, wild type (Wt), mutant (Mt), and complemented strains (Cp). Treatment used in the studied including indiwidual infiltration and co-inoculation.

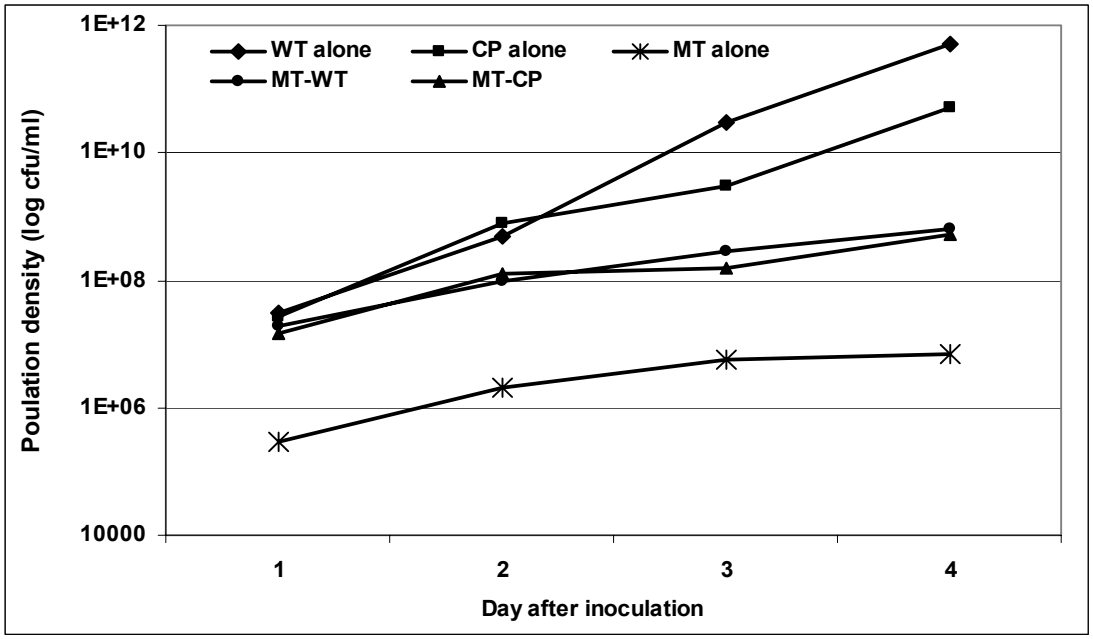

Figure 18 Growth of *X. axonopodis* pv. *glycines* strains on soybean leaves infiltrated with wildtype (WT), mutant KUMNTP2 (MT), and complement (CP) ; and co-inoculation with wildtype and complement on 24 h pretreated soybean plants with mutant strain (MT-WT, MT- CP) respectively.

In order to confirm if SAR is responsible for the protection effect of mutant against pathogenic Xag wildtype, symptom development after challenge with pathogen was tested. The disease incidence and severity observed at 7 and 14 days after challenge inoculation wildtype showed that bacterial pustule lesions on tested soybean leaves was decreased when the time of pre-inoculation with mutant was increased and revealed highest severity on soybean plants inoculated with wildtype alone. Bacterial pustule symptoms on soybean leaves pre-inoculated with mutant at 4 days before pathogen challenge inoculated revealed lowest severity, followed by treatment of 3, 5, 2 and 1 day pre-inoculated with mutant respectively. The inoculation with wildtype caused disease severity of 38.2 and 71.5 % of leaf areas infection at 7 and 14 days after inoculation respectively where the co-inoculation of pretreated mutant and post-treated pathogen revealed tenfold smaller leaf area infected than the corresponding wildtype. Mutant treatment confused a protein against pathogenic Xag wildtype in all treatments tested, based on number of pustule lesion (Table 19). These results suggest that the incubation period of soybean plants after pre-inoculation with mutant might be effect to the level of defense reaction by the accumulation of some defense-related enzymes and enhancing the ability of suppressing growth of wildtype on host plants.

Table 19 Effect of pre- inoculation with *X. axonopodis* pv. *glycines* (Xag) mutant on bacterial pustule reduction at different post inoculations with wildtype

Time of pre-inoculated mutant followed by post-inoculated wildtype (day) [1/]	Disease severity (%)[2/]	
	Day after wildtype inoculation	
	7	14
1	19.3 cd	37.8 c
2	18.7 c	32.4 c
3	16.8 c	23.8 bc
4	10.1 b	15.7 b
5	17.6 c	24.4 bc
Mutant alone	2.0 a	5.7 a
Wildtype alone	38.2 d	71.5 d
CV.	3.5	3.5

[1/] Foliar spray was inoculated onto soybean plants using hand-hold spray bottles (Prathuangwong, 1984). Suspension of Xag mutant KUMNTP2 was foliar spray on tested soybean leaves before inoculation with wildtype.

[2/] Diseases severity was expressed as percent leaf area infection with *X. axopodis* pv. *glycines* evaluated by the method of Prathuangwong *et al.* (1993) at 7 and 14 days after inoculation. Means in the column followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's New Multiple Rang Test.

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Discussion

Bacterial pustule caused by *X. axonopodis* pv. *glycines* (Xag) is an important disease that effect to limited soybean production in warm temperature and high moisture growing areas (Prathuangwong *et al.,* 1990 ; Prathuangwong *et al.,* 1996). The diseases can be severe especially when the soybean is 30-40 days old (Sinclair and Dhingra, 1975) and affected to both quality and quantity of soybean production. The prevalence and severity of these diseases vary considerably from year to year because of differences in weather patterns and cultivars of soybean (Prathuangwong *et al*., 1996 ; Vauterin, *et al.,*1995). The disease causes severe losses when the susceptible cultivars such as local SJ family is grown (Prathuangwong, 1984; Prathuangwong *et al.,* 1990; Prathuangwong *et al.,* 1996). There is a few success methods to control the disease and those remain problems of side effects. The understanding of genes involved in pathogenicity and virulent related factors of Xag and its process for recognition in host plants and some of the disease resistant reduction will likely lead to improve strategies for soybean bacterial pustule management.

Several factors which may contribute to the virulence of Xag have been reported, such as production of indole acetic acid and cytokinin (Millar,1955 ; Fett and Dunn, 1987), extracellular polysaccharides (Jones and Fett,1985), toxin (Hokawat and Rudolph, 1993), bacteriocins (Fett *et al.,* 1987) or cellulase and protopectinases (Hokawat and Rudolph, 1993). Furthermore, the proceed of disease development are involve in the several stage started with attachment, invasion, recognition, multiplication of bacterial cells, production of virulence factors, and symptom development. Among these stages, these is rather limited information available on bacterial multiplication in the compatible combination because this process has not been given much attention in host-parasitic interactions of Xag-soybean involved to their genetic controlling.

In this experiment, the two representative strains of Xag, BK01 and No.12-2 which show high capability to induce disease severity were used for mutagenesis by

Tn5 insertion. All of 2,580 mutant strains (transconjugant colonies) were succeed form random Tn5 inserted into strain No.12-2, whereas strain BK01 could not screened any colony from this method. It was indicated that transfer frequencies of Tn5 fragment were influenced to a large extent by the bacterial strain, mating conditions and physiological state of donor and recipient cells investigated. Therefore, transposable elements have become valuable mutagenic tools for genetic and molecular analysis in many different bacteria. The most widely used transposon in Gram-negative bacteria is Tn5, which transposes at high frequency, has relatively little target sequence specificity, and dose not share homology with genomic sequences of most bacterial species. The insertion of a transposon into the bacterial genome will disrupt genes and provide insight to their function. In this experiment were successful use of transposon to mutagensis Xag only strain No.12-2 with average frequency of transposition was 6 $x10^5$ cfu per recipient where as strain BK01 could not succeed that may be involve in the technical limitations.

A total of 2,580 transconjugant colonies obtained from Tn5 insertion were analyzed for their pathogenicity associated genes. The 4-representative mutant strains, including KUMNTP2, KUM00812, KUPJ124, and KUM408 were apparently lacked of the pathogenicity on soybean cotyledon and was deficient or delayed in pathogenicity by foliar spray inoculation on susceptible soybean cultivars (SJ4). The results indicated that all of 4-mutant strain was mutated in different gene position. Mutant strains KUM00812, KUPJ124, and KUM408 could induced disease symptom on tested plant, but the disease severity was lower than the disease severity from wildtype, Only mutant strain KUMNTP2 showed lowest disease severity in both of cotyledon and foliar bioassays. In the experiment mutant strain KUMNTP2 showed deficient pathogenicity when it was inoculted on susceptible soybean (SJ4) cultivar and it was also exhibited the deficient HR induction on tobacco These results suggested that the deficient pathogenicity and HR induction was effected by Tn5 insertion attached in genes involved their pathogenicity hrp genes.

Sequences flanking the Tn5 insertions data of clone pUXMNTP2 and pUBLNT2 derived from mutant strain KUMNTP2 was analyzed with NCBI and

FASTA database for similarity to available sequences of Xag and BLASTX for similarity to other sequences in the major databases. The results revealed that no *X. axonopodis* pv. *glycines* sequences similarity to sequences flanking Tn5 insertion. However, sequences flanking the Tn5 insertion were found to be similar to those in phosphoenalpyruvate syntase (*ppsA*) gene of *X.axonopodis* pv. *citri* strain 360 with 99% identity, follow by *X. campestris* pv. *mavacearum*, *X. oryzae* pv. *oryzae*, and *X. campestris* pv. *campestris* with 97, 94 and 91% identity respectively. Consequently to confirm the properties of *ppsA* to reduction virulent and infection of *X. axonopodis* pv. *glycines*, complemented strain was constructed and the characterizations of genotype and phenotype-related of *ppsA* to their pathogenicity were investigated.

There are many reports have shown that gluconeogenesis is require for virulence of a number of animal pathogens such as *Salmonella enterica* serovar Typhimurium (Allen *et al.,* 2000), *M. bovis* (Collins *et al.,* 2003), *Mycobacterium tuberculosis* (McKinney *et al.,* 2000), and *Candida albicans* (Lorenz and Fink, 2001). In the term of *ppsA* gene, there is a few information are involve in the pathogenicity of phytopathogenic bacteria. Only report of *X. campestris* pv. *campestris* strain 8004, which preliminary test and revealed that the Tn5 disruption on *ppsA*, a one of key gene in gluconeogenesis pathway of *X. campestris* pv. *campestris* resulting in the failure of the pathogen to grow in medium with pyruvate or C_4-dicarboxylates as the sole carbon source and a significant reduction its virulence. The results also suggested that *X. campestris* pv. *campestris* possesses only the malic enzyme-PpsA route for gluconeogenesis, which is required for virulence (Tang *et al.* 2005). The primary function of the glyoxylate cycle is to permit C_2 compounds to be converted to glucose through the gluconeogenic pathway. It has been reported that the glyoxylate cycle plays an important role in the pathogenesis of a number of pathogens (Wang *et al.,* 2003.) In this study, carbon utilization depended on growth rate of Xag wildtype, mutant, and complemented strains was analyzed. Glucose and sodium acetated (NaAC) were selected used in this experiment as the representative of sugar and non sugar compounds respectively. Both of glucose and NaAC (and derivative compounds, succinate, fumarate, malate, and pyruvate) are necessary to gluconeogeneis (Oh *et al.,* 2002.).

Mutagenesis in *ppsA* gene gave an affected phenotype of Xag mutant KUMNTP2 with reduced cell multiplication, extracellular enzyme secretion, EPS production, and affected secretion protein related to HR induction. This study also demonstrates that *ppsA* was required for gluconeogenesis and related to virulence of Xag for induced bacterial pustule disease on host plant and HR on nonhost plant tobacco respectively. Mutant strain KUMNTP2 showed deficient pathogenicity when foliar spray inoculation on susceptible soybean (SJ4) cultivar with normal cell concentration (10^8 cfu/ml) was investigated and also exhibited the deficient HR induction on tobacco when infiltrated with the same concentration, but was able to induce HR on tomato. The pathogenicity of mutant strain was recovered when the cell concentration was increased or mixed with 10% exogenous glucose (v/v). The results indicate that the *ppsA* is required for physiology and virulence, and might be involved to hypersensitive response and pathogenicity *(hrp)* genes in Xag.

The first intimation revealed the growth of mutant KUMNTP2 in a synthetic medium (M9) supplemented with glucose was normally but was unable to grow in M9 medium supplemented with NaAC, whereas wildtype and complementation strains were able to grow well in these two media. Indicating that the deficient *ppsA* function was related to carbon source or carbohydrate utilization and cell multiplication since the ability to acquire nutrients from the host is the one essential factor for a pathogen to establish the next an infection process. Phytopathogenic bacteria utilize an apparent strategies to access the nutritional haven afforded them in the intracellular spaces of plant tissues (Dangl, 1995). In *planta* experiment, the multiplication level of Xag mutant was significantly lower than wildtype and complementat strains at all tested periods. The multiplication of mutant strain on host plant was corresponded to result of bacterial growth in *vitro* and the secreted effector accumulation affected to severity reduction of bacterial pustule on host plant. Taken data suggest that gluconeogenic pathway encoded by *ppsA* is required for virulence and related to the reduced bacterial numbers of the mutant in host plants. The results correspond to previous report that the severity reduction of black rot disease of cruciferous crops caused by *X. campestris* pv. *campestris* was effected by the deficient of ability to uptake nutrients from host plants (Tang *et al.,* 2005). Thus, the ability to acquire nutrients from the apoplast is critically

important for it to cause disease. Disruption of the gluconeogenic pathway resulted in significant reduction both in multiplication in plant tissue and virulence of bacterial pathogen. However, there are no report before that the carbon utilization ability depended *ppaA* was effected to pathogenicity and virulence gene expresion of plant pathogenic bacteria including Xag.

Our phenotypic characterization of Xag *ppsA*-null mutant showed that a type II protein secretion system is an important virulence determinant in this bacterium. This is demonstrated cellulase activity as a virulence factor in Xag. In the absence of *ppsA*, the extent of lesion development was reduced on all methods or techniques investigated for Xag pathogenicity. Exogenous glucose added to the bacterial suspensions restored the full virulence of the *ppsA*-null mutant, further suggesting that phosphoenal pyruvate is the cause of the reduced lesion development in this mutant. The data were consistent with a role for *ppsA* in the pre-and post-penetration stages of the infection process that allowed the colonization of the host. One hypothesis that could be considered is that *ppsA* mutant develops more slowly in the host soybean plants tested because of stress imposed by insufficient gluconeogenesis supplied that impaired growth rate and amount of secreted protein effectors then, the pathogenicity impact. As the results have been shown, the effective potential of Xag, wild type and complementation strains for uptake some available carbon sources on tested soybean leaves might be higher than the mutant and could be enhanced development of bacterial cell from the epiphytic phase to pathogenic phase (Sigee, 1993). This results suggest that the mutant strain was deficient to use some carbon source on host plant and carbon diet of a pathogen inside the host is correlated to the composition of the available carbon source in the infection site. From evolutionary point of view, the nutritional requirements of a pathogen during infection and the molecular mechanism by which this pathogen acquires nutrients from the host may be the result of co-evolution of the pathogens with their hosts (Sigee, 1993).

The several factors which may contribute to the virulence of plant pathogenic bacteria including Xag have been reported, such as production of indole acetic acid and cytokinin (Fett and Dunn, 1987), extracellular polysaccharides (EPS), toxin,

bacteriocins or cellulase, xylanase and protopectinases (Sun *et al.,* 2005; Hokawat and Rudolph, 1993). In this study, the results showed that mutant KUMNTP2 was deficient to produce the cellulase and EPS that is the one of virulence factors of Xag. The lack of enzyme secretion and EPS production might involve in its virulence that effected by defective *ppsA* genes and quorum sensing. The results of this study could be considered the *pps*A genes are probable roles or involved in the virulence of Xag by effect to carbon utilization on host plant and capability to multiply in the infection areas, and involved in the virulent factor production such as cellulase and EPS. The disrupted *ppsA* function might be also direct or indirect effects for enzyme secretion channel or protein transport membrane. In this study, the decrease of cellulase production was effected from some problems of secretion pathway. The level of cellulase detected in periplasmic space of Xag mutant strain however, higher amount than that wildtype and complementation strains. The accumulation of cellulase in periplasmic space was relating to the transport membrane not function, and indicated that *ppsA* might be involved in protein transport membrane. In generally, type II secretion pathway is encoded by at least 12 genes and specifically supported the transport of a group of seemingly unrelated proteins across the outer membrane (Sandkvist, 2001). Proteins secreted by the type II pathway include proteases, cellulases, pectinases, phospholipases, lipases, and toxins. These proteins are associated with destruction of various tissues, which contributed to cell damage and disease severity. The previous report have shown that regulating the production of extracellular enzymes and EPS themselves are under quorum sensing control or are strictly regulated by the environment at the site of colonization (Poplawsky *et. al.,* 1998). However, *ppsA* in this study remains to determine whether it links to *rpf* encoded DS or DSF function of quorum sensing in Xag. Additionally, the EPS and EPS-encoding genes of genus *Xanthomonas* have been well characterized (Harding *et al.,* 1987; Reinhard *et al.*, 1992; Chou *et al.,* 1997). Polysaccharides of *X. campestris* pathovars were found to contain sugars such as glucose, mannose and glucuronic acid with pyruvate and acetate subunits (Coplin and Cook, 1990). Although the correlation between EPS production and virulence has been studied recently in *Pseudomonas* and *Xanthomonas* pathogens (Sutton and Williams 1970; Coplin and Cook 1990 ; Katzen *et al.,* 1998; Dharmapuri *et al.,*1999), but not yet in Xag on host plants soybean. We

showed this type of regulation results in controlled secretion of virulence factors of Xag that will only occur when the bacteria have reached their correct location and obtained a critical mass for success infection that compromised with previous report (Sandkvist, 2001).

Our experiment also found that *ppsA* could participate to Hrp effect that triggered cell death in different nonhosts. Xag mutant failed to induce HR on tobacco, but was able to induce HR on tomato at the cell concentration of 10^8 cfu/ml. The mutant strain recovered to induce HR on tobacco when the cell concentration was increased to $2x10^{11}$. The result suggests that bacterial density was effected in their ability to HR induction. Previous research on the HR elicited by Xag reported that this bacterium did not induce the HR on tobacco but efficiently causes the response on pepper and, in particular on tomato (Park and Hwang, 1999 ; Kim *et al*., 2003; Hwang *et al*.,1992). In contrast, Kaewnum *et al.* (2005) reported that some strains of Xag isolated from Thailand showed aggressive ability to cause HR on various tobacco cultivars when used cell concentration of 10^9-10^{11} cfu/ml. The ability of Xag to induction of HR on tobacco was related on the function of pactate lyase in which a one type of exoenzyme secreted by Xag (Kaewnum *et al.*, 2006). In this study however, we also demonstrated that Xag wildtype No.12-2 succeeded in HR elicitation on tobacco that was correlated with protein secretion affected by *ppsA* null-mutant. Consistent to these information, our result indicated that *ppsA* might be affected to HR induction on tobacco base on protein secretions involved in the number of cell multiplication. Interfere of *ppsA* was related to carbon source utilization and cell multiplication of Xag on tobacco and might be direct or indirect effect to expression of *hrp*-or *hrp* associated genes that depended on quorum sensing or biofilm formation (Poplawsky and Chun, 1997; Poplawsky *et al.,* 1998). Plant pathogenic bacterial cells sense their population density through a cell-cell communication system or quorum sensing with production of signaling and receptor molecules and triggered expression of particular genes when the density reached a threshold including exoenzymes, EPS, and *hrp* genes. (Tang *et al.,* 199; Barber *et al.,* 1997). These results suggest that *ppsA* disrupted may also be effected to the quorum sensing of Xag which an important roles in plant-bacterial interaction that remains to further study.

The *ppsA*mutant KUMNTP2 strain was deficient to secrete cellulase that is one of Xag virulent factor. The reduction of cellulase concentration was related to full virulence of Xag mutant. One possibility could be considered that the lower level of cellulase secreted by mutant strain might be played the roles of plant defense elicitors. Previous report revealed that some exoenzyme secreted by plant pathogenic bacteria such as *Erwinia carotovora* and fluorescent Pseudomonad, which secreted plant cell-wall degrading enzymes (CDEs) including pectate lyas, polygalacturonase, cellulase, and protease played significant rules on host-pathogen interaction as plant defense elicitor and HR induction when applied with optimum concentration. Pectinase and cellulase are able to induce a defense response both locally and systemically on host plants and nonhost plants (Vidal *et al.,* 1997, Vidal *et al.,* 1998) by a signal transduction (Vidal *et al.,* 1997). Consequently, the reduction of cellulase concentration was related to full virulence of Xag mutant. Our previous report have found the different sizes of necrotic lesions on soybean leaves infiltrated with different concentrated extracted cellulase of Xag. Among concentration of 100 % cellulase, and diluted concentration of 1:1 and 1:2 showed aggressiveness to induce cell necrotic within the same period of 3 days. These necrotic cells were disease lesions as shown by yellowing and browning of leaf vein and adjacent regions which was different from HR-like browning reactions. In lower concentrations of 1:3, 1:4 and 1:5, the initiate lesions showed slowly and slightly necrotic spots visible in infiltrated area observed in 5 days as a disease development of symptoms. This result could be confirmed that cellulase played a virulent factor for Xag to enhance disease severity and full virulence.

One of the interaction among pathogen and its host plant which negative disease development is that host plant could establish some defense response against pathogen invasion thoroughly the different models of SAR and ISR (Van Loon, 1997). The meaning of induced resistance is the evidence of silence of host resistance can be express when stimulates by some inducing agents (Kloepper *et al.,* 1992) including pathogens and their weakly virulent strains (by either elicitor molecules from the pathogens or by plant cell wall components released by the action of pathogens),

biocontrol agents, certain types of composts, and plant activating compounds (endogenous elicitors) (Van Loon and Van Strien, 1999).

Recent research indicated that one of elicitor molecules from the pathogens is cell-wall degrading enzymes secreted by bacterial pathogens which have been shown to be effective elicitors of plant defense response via the production of phytoalexins, lignin deposition and oxidative burst (Ryan and Farmer, 1991). The fraction of cell-wall degrading enzymes secreted by pathogenic bacteria have been reported to cause plant cell death (Braun and Rodrigues, 1993). Some exoenzyme secreted by plant pathogenic bacteria such as *Erwinia carotovora* and fluorescent Pseudomonad, which secreted plant cell-wall degrading enzymes including pectatelyase, polygalacturonase, cellulase, and protease played significant rules on host-pathogen interaction as plant defense elicitors and HR induction. Pectinase and cellulase are able to induce a defense response both locally and systemically on host plants and nonhost plants (Vidal *et al.,* 1997; Vidal *et al.,* 1998) by a signal transduction (Vidal *et al.,* 1997). Tobacco treated with pectinases and some fraction of cellulase as a triggering systemic resistance to the pathogen encoded by the β-1, 3 glucanase gene (Vidal *et al.,* 1998). Cell-wall degrading enzymes such as cellulase may play a dual role in plant resistance to pathogen that multiple reports exhibiting its involvement in the induction of defense reaction including HR cell death (Braun and Rodrigues, 1993). In the other hand, its accumulation showed evidence in successful pathogenesis of necrotropic pathogens like Xag experiments mentioned above (Kasem *et al.,* 2007). To assess whether bacterial exoenzymes is an effective elicitor to induce HR, cellulase extraction of Xag diluted in different doses was infiltrated in tobacco nonhost plant and the interaction was observed at 6 h interval. The result revealed that at the concentration of 2:1, 1:1 and 1:2, initial and clear HR-like browning spots were observed within 36 and 42 h after infiltration respectively. At 1:3 concentration, unclear HR-like induction was detected in 48 h whereas no interaction was formed on tobacco at the doses of 1:4 and 1:5 (data not shown). The earlier report however, we demonstrated that extracted Xag cellulase at these equivalent concentrations of 2:1, 1;1 and 1;2 induced severe yellowing- and browning- symptoms on soybean host plants at infiltrated sites and adjacent lesions in 3 days, whereas smaller sizes of yellowing area were detected in 5

days after soybean plant were infiltrated with 1:3, 1:4, and 1:5 (Kasem *et al.,* 2007). These works indicate that infiltration of balance concentrations of Xag cellulase elliciter resulted in either visible HR and evidence of cell damage, or linked to another pathway. In this study, at lower and higher concentrations, cellulase extracted from Xag culture filtrated (CMC-broth) may induce SAR without visible HR and visible cell damage that may result in the initiation of basal defense responses, such as callose deposition (Braun and Rodrigues, 1993). In the pathway of cell death initiation, production of cellulase facilitated the cell wall degradation, paving the way for the pathogens to colonize the host tissues and provide products which serve as pathogen nutrients, at the same time (Vidal *et al.,* 1998).

Whether the HR is caused directly by a protein product or by a metabolic synthesized by the bacteria discussed below (such as phytoalexins and PR-protein accumulation), remained to be determined. A cellulase extraction from Xag at equal concentrates induced both HR on nonhost and caused a yellowing and necrosis on host plants that conducted in this study suggested that its pathogenicity genes may encode an exocellular compound that is different from that major degradative enzymes (or different types of cellulaes secretion) and is required for both virulence on soybean host and HR on tobacco nonhost plants. It is still unclear whether the same component is actually responsible for both phenotypes.

To investigate Xag *ppsA* mutant linking to defective cellulase production that increased plant resistance against pathogenic Xag wildtype in soybean through a SAR mechanism, single and co-inoculation of either mutant, wildtype or complement strains on to soybean plants were conducted under greenhouse conditions and the defense related enzymes and chemicals were measured. Soybean plants treated with mutant KUMNTP2 before challenged with pathogenic Xag wildtype were significantly greater amount accumulation of β-1,3 glucanase and phenylalanine ammonia lyases (PAL), where the activity of phenolic compounds and peroxidase (POX) were exhibited in the equivalent levels of all treatments investigated. The β-1,3 glucanase and PAL were found to accumulate in non-pathogenic mutant leaf tissues at

1 day after pathogen challenged and reached maximum at 3rd and 4th day respectively. In pathogen inoculated plants, the accumulation of β-1,3 glucanase and PAL started at the 2nd and reached maximum at 3rd and 4th then, drastically decreased at 4th and 5th day after the pathogen inoculation respectively. The accumulation of these enzymes could be comprised stress signaling molecules such as salicylic acid (SA) and jasmonic acid (JA), expression of proteins with antimicrobial activities involved in synthesis of phytoalexins and PR-proteins (Bowles 1990; Somssich and Hahlbrock, 1998). This result confirmed that the mutant KUMNTP2, the deficient cellulase secretion strain played the activity as defense – elicitor to induc expression of defense-related enzyme genes especially β-1,3 glucanase and PAL. The β-1,3-glucanases have been well investigated in plants at the physiological and molecular levels due to their widespread role in plant defense responses (Meins *et al.,* 1992; Simmons, 1994). It has been reported that this enzyme is expressed constitutively at low levels, and is secreted into the cell wall and intercellular spaces where it encounters the invading pathogen (Krishnaveni *et al.*, 1999). The report of soybean plants inoculted with the bacterium *Pseudomonas syringae* pv. *glycinae* or with a fungal elicitor from *Phythophtora* spp. has been found to increase expression of a basic class III isoform of β-1,3-glucanase (Cheong *et al.,* 2000). Similar studies have been reported in tomato (Roulin and Buchala, 1995), chickpea (Vogelsang and Barz, 1993), and potato. A few studies reported on the induction of β-1,3 glucanase activity in response to wounding and other stress stimuli (Beerhues and Kombrink, 1994). These works were corresponded to our results that β-1,3-glucanase activity of soybean showed average increased at 0.3-fold when co-inoculation with Xag mutant together with wildtype or complemented strains compared with individual inoculation. In this case could be considered that β-1,3-glucanase is an affective elicitor molecule to enhance defense-protein expression, such as PR proteins. Recently, eleven families of *PR* genes (*PR-1* to *PR-11*) in crop plants have been identified (Van Loon *et al.,* 1994). These *PRs* were classified in at least nine plant family which mainly characterized in tobacco and tomato. The family of these *PRs* was identified base on PR-protein products including 4 families of chitinases (PR-3,-4,-8, and -11), one of β-1,3-glucanase (PR-2), one of protein inhibitor (PR-6), and one specific peroxidase (PR-9), where the function of PR-1, PR-5, and PR-10 was difference (Van Loon, 1997). These information indicated

that the accumulation of β-1,3-glucanase in soybean treated with Xag mutant was involved in PR-2 of SAR pathway and also suggested that the defense response of soybean against bacterial pustule induced by nonpathogenic mutant depended on expression of β-1,3-glucanase encoded by *PR2* genes. This recognition triggers indicated the defense responses may be however, induced specifically, as in a gene for-gene type of interaction (Flor 1971; Keen 1990; Staskawicz et al. 1995), or nonspecifically by a range of biotic and abiotic elicitors produced by the microorganisms (Darvill *et al.*, 1992).

Moreover, our result indicated that phytoalexin production also involved in the defense mechanism of soybean plants against attack by virulent strain of Xag since the accumulation of PAL was significantly increased in soybean plants treated with Xag mutant. The present observations that all soybean pre-treated plants with Xag mutant showed higher accumulation level of PAL than individual inoculation with wildtype and complement strains. The accumulation of PAL was found in non-pathogenic mutant leaf tissues at 1 day after pathogen challenged and reached maximum at 4^{th} day. This result was related to previous reported that the phytoalexin production in several defense responses against attack by pathogens is regulated at the accumulation level of the enzymes PAL, chalcone synthase (CHS), and chalcone isomerase and regulating the expression of the initial enzymes of the phenylpropanoid pathway (Dixon and Paiva, 1995). PAL is the key enzyme in the production of phytoalexins in plants. For total phenols and POX activities detected, they were accumulated in the equivalent levels of all treatments investigated in this study, indicated that Xag *ppsA* mutant might be not contributed to enhance the mechanism strength of host cell wall and did not inhibit the bacterial growth as these chemicals are toxic in nature (Dixon and Paiva, 1995). Neither mutant nor wildtype of Xag were then, strong inducers of phenolics and POX. Xag ppsA mutant therefore, screened to trigger specific PR-2 and HR indiction of SAR pathway that corresponding to either β-1,3-glucanases or PAL accumulation. The accumulation level of β-1,3 glucanase and PAL in different treated soybean plants was related to the severity of pustule symptoms. Soybean plant inoculated with wildtype and complement alone showed disease incidence within 4 days after inoculation (data not show) and presented clearly disease severity at 7 days

after inoculation with 59.7 and 39.7 % area leaf infected respectively. Whereas soybean treated with mutant showed a slightly disease severity. Co-inoculation with mutant and wildtype ; and mutant and complement strains showed lower disease reduction when compared with individualy inoculated with wildtype and complement strains alone. This result suggests that the accumulation of β-1,3-glucanase and PAL induced by Xag mutant was effected to defense response eliciting on host plants and suppress bacterial colonization, and symptom expression in host plant tissues, consistent with a role of either PR-protein (PR-2) and phytoalexins in the acquired resistance pathway regulated by either β-1,3-glucanase and PAL respectively. The relatively growth rate of Xag wildtype was also related with disease severity. The disease incidence was observed at 24, 48, 72 and 96 h after individual or co-inoculation. Treatments infiltrated by wildtype alone and co-inoculation with mutant strains showed different cell multiplication rates on treated soybean leaves. Population density of wildtype was rapidly grown in soybean leaf tissues when inoculated alone, whereas the multiplication of wildtype was lower when it was inoculated after 24 h mutant treated. The growth reduction of pathogenic Xag wildtype in soybean host plants may derive from the suspension effects of enzyme defense response on Xag *ppsA* mutant and the phytoalexin and PR protein production. The plant inoculation experiments were consistent with the proposed defense role of these plant defense chemicals in, as the severity of symptoms produced was correlated with sensitivity to these chemicals that the co-inoculation showed milder symptoms than the single Xag wildtype inoculation. The earlier and higher accumulation of enzymes involved in phenylpropanoid metabolism and PR-proteins regulated by accumulation of β-1,3-glucanase and PAL found in soybean leaf tissues treated with mutant strain KUMNTP2 were responded to invasion of Xag wildtype. The plant-pathogen interactions have also triggered the activities of defense enzymes initially but later the activities drastically declined when the pathogen colonized the leaf tissues. Accumulation of β-1,3-glucanase and PAL induction increased in soybean leaf tissues might be a signaling pathway contributed to induced resistance in soybean plants against Xag wildtype.

However, it should be noted that Xag *ppsA* mutant linked to defective cellulase production could indeed exhibit enhanced resistance toward pathogenic Xag wildtype upon SAR pathway. Our results are in agreement with Kamoun and Kado (1990) who reported that some cellulase and EPS secreted by *X. campestris* pv. *campestris* were not involved in induced host defense response (Kamoun and Kado, 1990**).** In this study, cellulase produced by Xag may not be effective or necessary in defending the pathogen and it is not important for the Xag *ppsA* mutant induced protein against pathogenic Xag wildtype in soybean host plants. The defense response induced by their Xag mutant is still not clear and not due to direct recognition of cellulase or TIIS effectors. Although we selected this mutant that demonstrated that affected to TIIS on inducer, we cannot exclude the possibility that the effect observed with their mutant is not due only to this cellular production. However, SAR pathway activated is varied with the type of pathosystem and host tissues which renders the application of this resistance induction in crop plants less predictable (Van Loon, 2000). The pattern of defense in induction and growth in plants involved in these defense pathways for Xag mutant and wildtype indicated that the genes involved in plant-bacteria co evaluation are likely to be as diverse as the pathogen themselves. In conclusion, growth of wildtype *in planta* and the quantification of defense related enzymes provide evidence likely to contribute to the generate basic for variation in resistance and virulence in this interaction that also mutant induces and wildtype is suppressed by related enzymes mediated defense response.

CONCLUSION AND RECOMMENDATION

Conclusion

From the experimental results and discussion of this study, the conclusion can be drawn as follow:

1. *Xanthomonas axonopodis* pv. *glycines* (Xag) mutant strain KUMNTP2 obtained from Tn5 mutagenesis showed deficient in several phenotypes including reduced cell multiplication, extracellular enzyme secretion, EPS production, and affected secretion protein related to HR induction. The mutant strain showed complete deficient pathogenicity and HR induction when foliar spray inoculation on susceptible cultivar (SJ4) of soybean plant and infiltrated in nonhost plant tobacco respectively.

2. Nucleotide sequence analysis within the Tn5 flanking insertion in the chromosomal DNA of mutant KUMNTP2 showed high significant homology with *ppsA* geneas of *X. axonopodis* pv. *citri* (99%), *X. campestris* pv. *mavacearum* (97%), *X. oryzae* pv. *oryzae* (94%), and *X. campestris* pv. *campestris* (91%) respectively.

3. In *vitro* experiment, growth of mutant KUMNTP2 in a synthetic medium (M9) supplemented with glucose was normally but was unable to grow in M9 medium supplemented with NaAC, where as wild type was able to grow well in those medium as the complement strain, In *planta* test, mutant was deficient pathogenicity and HR induction when it was inoculation with normal cell concentration (10^8 cfu/ml) on susceptible soybean (SJ4) cultivar and tobacco respectively. The pathogenicity and HR induction was recovered when the cell concentration was increased or mixed with 10% exogenous glucose (v/v). Indicate that deficient of *pps*A function was related to carbonsource utilization and cell multiplication and the *ppsA* is required for physiology and virulence in Xag.

4. The exoenzyme secretion assays revealved that cellulase production and secretion requires induction from *ppsA* gene. Xag mutant have been decreased level of

cellulase production whereas wildtype and complement strains were clearly secreted cellulase in tested plates. Most of the cellulase activity of mutant strain were detected in the periplasmic fraction and very few in the extracellular fraction of mutant strain when the wildtype and complement shrains showed high activity of its extracellular expression, whereas periplasmic and cytoplasmic fractions had very little activity.

5. For EPS production, the amount of EPS produced by the *ppsA* mutant was six fold lower than that of wildtype cells that it could be detected. This suggests that reduction of EPS can compensate for absence of some signalling protein in EPS synthesis and supports the idea that *ppsA* affects the production of EPS via the lower cell multiplication and cell-cell communication.

6. One possibility could be considered that the lower level of cellulase secreted by mutant strain might be played the roles of plant defense elicitors. Soybean plants treated with mutant KUMNTP2 before challenged with pathogenic Xag wildtype were significantly greater amount accumulation of β-1,3 glucanase and PAL, where the activity of phenolic compounds and POX were exhibited in the equivalent levels of all treatments investigated. The β-1,3 glucanase and PAL were found to accumulate in non-pathogenic mutant leaf tissues at 1 day after pathogen challenged and reached maximum at 3^{rd} and 4^{th} day respectively. The accumulation of these enzymes could be comprised stress signaling molecules such as salicylic acid (SA) and jasmonic acid (JA), expression of proteins with antimicrobial activities involved in synthesis of phytoalexins and PR-proteins

7. The accumulation of β-1,3 glucanase and PAL induced by Xag mutant was effected to defense response eliciting on host plant and suppress bacterial colonization, and symptom expression. Soybean plant inoculated with wildtype and complement alone showed aggressive disease incidence whereas soybean treated with mutant showed a slightly disease severity. Co-inoculation with mutant and wildtype ; and mutant and complement strains showed lower disease reduction when compared with individualy inoculated with wildtype and complement strains alone.

8. The relatively growth rate of Xag wildtype was also related with disease severity. Population density of wildtype was rapidly grown in soybean leaf tissues when inoculated alone, whereas the multiplication of wildtype was lower when it was inoculated after 24 h mutant treated. The growth reduction of pathogenic Xag wildtype in soybean host plants may derive from the suspension effects of enzyme defense response on Xag *ppsA* mutant and the phytoalexin and PR protein production.

9. The defense response induced by their Xag mutant is still not clear and not due to direct recognition of cellulase or TIIS effectors. Although we selected this mutant that demonstrated that affected to TIIS on inducer, we cannot exclude the possibility that the effect observed with their mutant is not due only to this cellular production. However, SAR pathway activated is varied with the type of pathosystem and host tissues which renders the application of this resistance induction in crop plants less predictable.

Recommendation

More experiments will be necessary to proved a cause-effect relation between increased defense chemical sensitivity and avirulence of pathogens. Also, the agreement or conflicting observations with the results of exoenzymes produced by phytopathogenic bacteria, was involved in triggering host defense response, the mechanism of their elicitor in nit clear and remains to be reconciled. The mechanisms responsible for their increased resistance are unknown in this Xag. Next experiment, using the model that Arabidopsis thaliana provided the advantage of mutants in different pathways elucidation the mechanism contributed to resistance, is under taking in our laboratory. However, further work is needed also to identify additional virulent factors and effective ellicitoes to defense response of the Xag strains.

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APPENDIX

1. Recipes of media

1.1 Nutrient glucose agar (NGA) and nutrient broth (NGB)*

	per L
Beef extract	3.0 g
Bacto peptone	5.0 g
Glucose	2.5 g
Agar	15.0 g

Nutrient agar (NA) or nutrient broth may be purchased in dehydrated form from Difco, * Do not add agar if nutrient broth is desired.

1.2 Nutrient-broth yeast extract agar (NBY) and nutrient-broth yeast extract broth*

	per L
Nutrient broth	8.0 g
Yeast extract	2.0 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
Glucose	2.5 g
Agar	15.0 g

After autoclaving, add 1.0 ml of a sterile solution of 1M $MgSO_4$. $7H_2O$, * Do not add agar if nutrient broth is desired.

1.3 Modified medium for *Xanthomonas campestris* pv. *glycines* (MXG)

Basal media

	per L
K_2HPO_4	0.8 g
KH_2PO_4	0.6 g
Yeast extract	0.7 g
Glucose	2.5 g

Agar	15.0 g
Methly green solution	1.0 ml
(1% aqueous solution)	

After autoclaving aseptically add the following antibiotics:

Cyclohexamide	1.0 ml (50 ppm)
Bacitracin	0.2 ml (10 ppm)
Cephalexin	1.0 ml (50 ppm)

1.4 Luria bertani (LB) agar and Luria bertani broth *

	per L
Bacto typtone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Agar	15.0 g

Do not add agar if nutrient broth is desired.

2. Diagnostic media

2.1 Motility Test medium (pH 6.8 – 7.0)

	per L
Bacto-tryptone	10.0 g
Yeast extract	5.0 g
Agar	5.0 g

Motility agar is prepared in tubes without slanting. Dissolve in 1 L water and adjust to 6.8-7.0. Inoculate the sterile semi-solid medium by stabbing the center of the medium as above for geletin. Incubate and observe after 8,24, and 48 h. Motility is hown by a diffused zone of growth spreading from the line of inoculation. Diffuse growth may extend throughout the entrie medium or only from one or two points.

2.2 Starch Agar (Starch hydrolysis)

	per L
Bacto-tryptone	10.0 g
Yeast extract	5.0 g
Soluble starch	10.0 g
Agar	5.0 g

After incubated bacteria culture for 3-5 days drop the iodine solution and check the clear zone.

2.3 Catalase test

Mix a loopful of a 48-h-old culture grown on nutrient agar slant with a drop os freshly prepared 3% H_2O_2 . The formation of gas bubbles in indicates a positive reaction.

2.4 Hydrolysis of Gelatin (gelatin liquefaction)

	per L
Bacto-extract	10.0 g
Bacto-peptone	5.0 g
Glucose	2.5 g
Gelatin	12.0 g

Adjust the pH to 7.0. Add 10 ml to each tube, autoclave, and cool (do not slant) immediately. Inoculate by stabing a loop with inoculum into the center of the medium, then incubate the tubes a 20-22C or a room temperatur. Observed weather rapid or slow liquefaction occurs. Also observe shape produced when gelatin begins to liguefy.

2.5 Levan formation*

	per L
Beef extract	3.0 g
Bacto peptone	5.0 g
Glucose	2.5 g
Agar	15.0 g

*NA add 5 % of sucrose

2.6 Salt tilerance medium test*

	per L
Beef extract	3.0 g
Bacto peptone	5.0 g

* NB add 5, 7 and 10% of NaCl.

2.7 Carbon source utilization

Carbon sources are filter sterilized and add at 0.1%(w/v) final concentration to autoclaved and cooled Ayer et al. mineral salts medium.

	per L
$NH_4H_2PO_4$	1.0 g
KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
Peptone	1.0 g
Bromthylmol blue (1.6% v/v in 95% ethanol)	1.0 ml
Agar	1.5 g

* carbon source: Glucose,Dextrose,Fructose,manitol and maltose.

The pH is adjust to 7.1 prior to autoclaving. Bacteria are streaked onto the medium, or patched on with replica methods, and incubated at 27°C

2.8 Gram's crystal violet

Solution A

Crystal violet	2.0 g
95% Ethanol	20.0 g

Solution B

Ammonium axalate	0.8 g
Water	80.0 ml

2.9 Safranin solution

Safranin	2.5 g
95% Ethanol	100.0 ml
Water	100.0 ml