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THESIS

THE FLAGELLA SECRETION SYSTEM ON VIRULENCE OF SOYBEAN PLANT BY *Xanthomonas axonopodis* pv. *glycines*

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Xanthomonas axonopodis pv. *glycines* (Xag) causes bacterial pustule disease, resulting in severe losses in many soybean growing areas around the world including Thailand. Flagellum has been identified as one of structural components of virulent protein translocation into plant cells for bacterial infection, but not yet for Xag. Also, a recent study has demonstrated that a pectate lyase (Pel) in *xagP* of Xag 12-2 is required to induce a hypersensitive response (HR) on tobacco nonhost plant (*Nicotiana rustica*). Until now its role in pustule disease was unknown and whether the coordinate genetic regulation between flagellum biosynthesis and Pel expression was injected into target soybean host. To better understand of these functions, a genetic and characterization of the pectate lyase (*xagP*), flagella hock (*flgC*), flagellin (*fliC*), and hock associated protein 2 (*fliD*) genes of Xag strain 12-2 were carried out. Mutations in flagella genes *flgC, fliC,* and *fliD* resulted in loss of or reduced motility, biofilm formation, development of pustule disease and pathogen growth on soybean. In addition a pectate lyase gene (*xagP*) mutant also showed reduction in biofilm formation and virulence on soybean. The *xagP* mutant lost pectolytic activity whereas the *flgC, fliC,* and *fliC&fliD* expressed reduced pectolytic activity on potato slices suggesting the *xagP*-encoded pectate lyase may be secreted through flagella machinery. The flagella mutants were also altered in cellulase, alpha-amylase, endoglucanase, and protease activities again suggesting a role of flagella in secretion of these virulence factors. Complementation of mutants restored all phenotypes to wildtype levels. The flagella gene mutants did not affect development of a hypersensitive response on tobacco whereas the *xagP* mutant was HR-negative. These evidence indicated that flagellum was due in part to secrete pectate lyase resulting disease in soybean development. Since *fliC* and *xagP* decreased three-extracellular enzyme production, RT-PCR and NanoDrop analyses were investigated to examine the gene expression of alpha-amylase, cellulase, and protease. As expected, the lower mRNA levels detected in mutant than wildtype was observed. The relatedness of these genes was focused with two sigma factors *rpoE* and *rpoN* that revealed their responses to extracellular enzyme reaction and pathogenicity regulated by *xagP* and flagellin genes. The expression of *xagP* and *fliC* in *rpoE* and *rpoN* mutants was also reduced in cellulase, alpha-amylase, endoglucanase, and protease; and disease development, suggesting that the two sigma factors (XagPs and FliCs) were also essential for full virulence in Xag 12-2. This is the first report linking a production of effector proteins and their secreted function by these genes to bacterial pustule disease in soybean.

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Student's signature Thesis Advisor's signature

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THE FLAGELLA SECRETION SYSTEM ON VIRULENCE OF SOYBEAN PLANT BY *Xanthomonas axonopodis* pv. *glycines*

INTRODUCTION

Bacterial pustule caused by *Xanthomonas axonopodis* pv. *glycines* (Xag) is one of the major bacterial diseases of soybean in Thailand (Prathuangwong and Amnuaykit, 1989). Yield losses of soybean up to 40% have been reported in certain parts of the world (Prathuangwong, 1983). *X. axonopodis* pv. *glycines* produces a variety of pathogenicity factor including the type III secretion system (T3SS) (Thowthampitak *et al.*, 2008; Athinuwat *et al.*, 2009). Athinuwat *et al.* (2009) reported that *X. axonopodis* pv. *glycines* cells are motile in disease plant tissue, but whether the association of secondary metabolite production and secretion systems that contribute to virulence is coordinated with motility is unknown. As yet flagella genes have not been shown to contribute to virulence for this *X. axonopodis* pv. *glycines*. Bacterial cells have appendages for motility including flagella and pili that confer movement in liquid (swimming) and on solid surfaces (twitching) respectively (Mattick, 2002; O'Toole and Kolter, 1998). The motility of pathogenic bacteria on host plants may be essential for the initiation of infection and play roles in disease progression. Some bacteria are limited to colonizing the water-conducting xylem vessels of plants and may develop biofilms that contribute to the blockage of sap flow resulting in plant stress and disease (Maxwell *et al.*, 2003; Meng *et al.*, 2005; Li *et al.*, 2007). Flagella are complex organelles containing a built-in export apparatus of the hook and filament components whose synthesis depend upon approximately 50 gene products (Wolfe and Visick, 2008). In several animal host systems, bacterial motility is an important virulence factor mediating adhesion, invasion, and colonization (Pfund *et al.*, 2004). Moreover, evidence is beginning to accumulate that component of the flagella apparatus participate in the export of virulence determinants in several pathogens that closely related to T3SS. Most components of T3SS are conserved in both flagella and virulence-associated T3SS and many proteins are homologs in sequence or function (Abby and Rocha, 2012). The T3SS allows bacteria docked at the surface of a cellular membrane, to deliver effector proteins into the host cells (Abby and Rocha, 2012). In addition of flagella function, it is possible that like in other bacteria, the *X. axonopodis* pv. *glycines* flagellin may also elicit an hypersensitive response (HR) compatibility or incompatibility reaction on certain plants and induce a defense response. Plant-pathogen interactions including the HR and disease are often affected by three classes of bacterial proteins that include harpins, avirulence proteins, and pectic enzymes. Pectate lyase (*pel*) isozymes cleave α-1,4-galacturonosyl linkages in plant cell wall pectic polymers, resulting in tissue maceration and cell death due to osmotic fragility. Although Pels have been shown to play significant roles in pathogenesis of certain bacterial pathogens, their role in HR induction has not been demonstrated until in Xag 12-2 (Kaewnum *et al.*, 2005). They demonstrated that Pel encoded by *xagP* of strain 12-2 is required to induce a HR on tobacco nonhost plant (*Nicotiana rustica*). The role of *xagP* from Xag 12-2 on disease development of soybean bacterial pustule has however not been described. As a primary function, Pels is one of pathogenicity factors produced by almost phytopathogenic bacteria (Lei *et al.*, 1987; Nasser *et al.*, 1993; Debing *et al.*, 2006), other associated proteins and the T3SS regulated by flagellum also need to be elucidated for full virulence in Xag 12-2. In some bacterial species, the same proteins that regulate expression of flagella genes also regulate virulence factors. They may coregulate flagelin and the T3SS that is down regulated by *fli* genes or *fli* genes upregulates the T3SS (Athinuwat *et al.*, 2009). These differences show the regulatory effects of Fli secretion on virulence that cannot yet be predicted solely based upon comparison with regulated genes, rather, they must be experimentally determined (Athinuwat *et al.*, 2009). An overall picture of denitrifying *fli* gene and proteins involved in virulence remain to be developed. Potential participants in development are identified based on RNA expression patterns to show important roles through the application of gene mutation. *X. axonopodis* pv. *glycines* mutant transcriptomes on either uni-or double gene set differentially exhibited changes in mRNA including potential regulator of cellular processes, metabolic enzymes, structural proteins, and virulence factors are also elucidated.

The ability of bacteria to cause disease in susceptible plants (compatible interaction) and hypersensitive response (HR) in resistant plants (incompatible interaction) is elicited by various classes of virulence proteins including XagP (Kaewnum *et al.*, 2005). The HR is characterized by the rapid death of cells in the local region surrounding an infection, by producing reactive oxygen species (ROS) like superoxide radical, singlet oxygen, alkoxyl radical, superoxide anions, hydrogen peroxide (H_2O_2), hydroxyl radicals and nitrous oxide (Khan and Panda, 2002; Panda, 2002). These compounds affect cellular membrane function, in part by inducing lipid peroxidation and by causing lipid damage. The antioxidative system in plants, as one of the first defensive barriers, is set to sustain and extenuate the effects of ROS after the pathogens infection. Superoxide dismutase (SOD) is the main components of the cell antioxidative system. They catalyze the $H_2O_2^-$ dependent oxidation of a wide variety of reducing substrates, such as pyrogallol or guaiacol (Mittler *et al.*, 2004). These evidences are controlled by a cluster of *hrp* (hypersensitive response and pathogenicity) genes. Most *hrp* genes are involved in the assembly of protein secretion pathway, The Hrp pathway through which the virulence proteins lie across the bacterial envelope. Mutation of either *xagP* or *fli* genes affected activity of other virulence proteins such as extracellular enzymes that resulted in triggering. HR and disease are unknown in Xag 12-2.

Little is known about hypothetical interactions among secretion and production of the virulence proteins involved. The expression of virulence factors and motility of bacteria is intimately linked by an as yet uncharacterized mechanism. Several lines of evidence indicate that the activity of the flagellum of *Vibrio cholerae* might have an impact on virulence gene regulation, as alterations of the motility phenotype, either by mutation or by inhibitory drugs, result in varied levels of virulence factor production (Hase, 2001). No relationship has however, been established between various virulence proteins and the presence of flagellum in the soybean plant -Xag interaction. The initial success of bacteria to induce HR or disease includes not only virulence-associated genes (such as *xagP* and *fli* gens) but also genes involved in stress responses, polysaccharides synthesis, nutrient uptake, amino acid assimilation, and/or carbon metabolism (Milton and Saier, 1998). Among these gene groups, sigma factors encoded by *rpo* families have been shown to contribute to the virulence of multiple bacterial species (Dartigalongue *et al.*, 2001). This is essential to be elucidated whether sigma factor genes associate with *xagP* and *fli* genes in addition to virulence of Xag on soybean plants. However, *rpo* recombinant proteins heterologously expressed by bacterial differ in various aspects including specific activity, substrate preference, and mode of action (Dartigalongue *et al.*, 2001). The specific investigations were them studied the possible interactions between, *xagP* and *fli* genes in the elicitation of HR and disease and in abolishment of some extracellular enzyme activities. Address at the links among these evidences whether *rpo* genes also expressed was also conducted.

OBJECTIVES

The objectives of this research were the following:

1. Identification of Xag full virulence on soybean is regulated by flagella genes (*flgC, fliC* and *fliD)* and pectate lyase (*xagP)* gene.

2. Study on pectate lyase encoded by the *xagP* in *X. axonopodis* pv. *glycines* 12-2 whether it could be secreted via flagellum and promote the virulence of bacterial pustule disease on soybean.

3. To complement these investigations into the role of sigma factor regulation.

LITERATURE REVIEW

1. Soybean (*Glycine max*)

Soybean is a leguminous vegetable of the pea family that grows in tropical, subtropical, and temperate climates. Soybean was domesticated in the 11th century BC around northeast of China. It is believed that it might have been introduced to Africa in the 19th century by Chinese traders along the east coast of Africa (Ashaolu and Noibi, 2013; Endres, 2001).

1.1 Importance of soybean

It consists of more than 36% protein, 30% carbohydrates, and excellent amounts of dietary fiber, vitamins, and minerals. It also consists of 20% oil, which makes it the most important crop for producing edible oil. Malnutrition, particularly protein deficiency, is prevalent in many parts of Africa as animal protein is too expensive for most populations (Adekunle *et al.*, 2006). Many leguminous crops provide some protein, but soybean is the only available crop that provides an inexpensive and high quality source of protein comparable to meat, poultry and eggs. A by-product from the oil production (soybean cake) is used as a high-protein animal feed in many countries. Soybean also improves soil fertility by adding nitrogen from the atmosphere. This is a major benefit in African farming systems, where soils have become exhausted by the need to produce more food for increasing populations, and where fertilizers are hardly available and are expensive for farmers (Ashaolu and Noibi, 2013).

1.2 Production of soybean

More than 216 million tons of soybeans were produced worldwide in 2007, of which 1.5 million were in Africa. Africa imports nearly as much soybean as it produces. Africa exports about 20,000 tons annually. Nigeria is the largest producer of soybean in sub-Saharan Africa (SSA), followed by South Africa. Low yields (<1 t/ha in tropical Africa) and a shortage of fertilizer constrain the ability of some countries to increase production. In Nigeria the haulms and post-processed pulp (soybean meal) serve as important sources of animal feed. A 30% annual growth in the poultry industry from 2003 to 2008 fuelled such a demand for soybean meal that an increase in imports was required. Commercial soybean production on large farms takes place in Zambia, Zimbabwe and South Africa. However, it is mostly cultivated by small-scale farmers in other parts of Africa where it is planted as a minor food crop among sorghum, maize, or cassava (Shurtleff and Akiko. 2013).

1.3 Harvesting

Nearly 95 million hectares of soybeans were harvested worldwide in 2007, with 19 million in Asia, 3.5 million in the USA, and 1.2 million in Africa. Depending on the variety, soybeans can be harvested between 100 and 150 days after planting. Labor requirements in Africa are high since most cultivation and harvesting are done manually (Ashaolu and Noibi, 2013; Endres, 2001).

1.4 Consumption

Worldwide consumption of soybean is nearly 11 million tons. Africa consumes about 618,000 tons annually, and uses another 4,800 tons for animal feed. Nigeria is the largest consumer of soybeans in SSA followed by Uganda. In Africa dry soybeans are used to produce milk substitutes and flour. The bean curd is fried and eaten as a snack or breakfast food. Mature beans are not easily digested and contain toxic compounds, which require soaking and prolonged cooking (Ashaolu and Noibi, 2013; Endres, 2001).

2. 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). Utilization of soybean provides an important protein source in the diets of many Asian nations and is an extremely valuable food and industrial product. 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 cultivars. Soybean has been planted since 2503 B.C. in Thailand. The important soybean growing areas are 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 SJ4, CM60, KKU35 and Rachamongkol1 which are planted in different areas. However, soybean production in Thailand is not enough for both human consumption and use in animal feed materials. Total soybean seed production is approximately 500,000 tons / year whereas the total demand of seed is more than 600,000 tons. Diseases caused by several pathogens including fungi, bacteria and virus are the main problems in soybean production (Prathuangwong *et al.*, 1996; Prathuangwong *et al.*, 2009). Important diseases of soybean in Thailand include (1) Sudden death syndrome caused by *Fusarium solani*, (2) Antracnose (*Colletotrichum truncatum*), (3) Rust (*Phakopsora pachyrhizi*), (4) Downy mildew (*Peronospora manshurica*), (5) Bacterial pustule (*X. axonopodis* pv. *glycines*), (6) Crack stem (*Fusarium* spp., *Phialophora* sp., and *Rhizoctonia* sp.) (7) Phyllosticta leaf spot (*Phyllosticta* sp.), (8) Myrothecium leaf spot (*Myrothecium* sp.), (9) Rhizoctonia aerial blight (*Rhizoctonia* sp.), (10) Yellow orange leaf spot (unidentified) (11) Basidiomycete rot (Basidiomycete fungus), (12) other fungal foliar pathogens (*Alternaria* sp., *Cercospora* sp., *Curvularia* sp., and *Septoria* sp.), (13) seedling pathogens (*Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Stylopage* sp., and *Rhizoctonia* sp.), (14) unidentified diseases, and (15) other causes of decline (symptom like physiogenic, mineral deficiency, mutative disease, virus and phytoplasma) (Prathuangwong and Amnuaykit, 1998; Prathuangwong *et al.*, 1993) which negatively affect commercial production in Thailand. The disease distribution and severity observed of diseases are affected by the different climates of the soybean growing regions (Prathuangwong *et al.*, 1993).

3. Soybean bacterial pustule and causal pathogen

Bacterial diseases of soybean occur worldwide and severely limit production during years of warm temperature and high moisture. The most common bacterial diseases are bacterial blight and bacterial pustule. The prevalence and severity of these diseases vary considerably from year to year and are dependent on the differences in weather patterns and cultivars grown. Bacterial pustule caused by *X. axonopodis* pv. *glycines* (Parathuangwong, 1983; Prathuangwong and Kasem, 2004; Sinclair and Backman, 1982) occurs worldwide but is more severe in moderate to high temperature regions. The disease is important in many 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, 1983; Prathuangwong *et al.*, 2005; Prathuangwong *et al.*, 2008). Soybeans are especially susceptible when 30-40 days old (Prathuangwong, 1983) and the disease affects both quality and quantity of soybeans.

Initial symptoms of bacterial pustule are small, yellow-to-brown lesions with raised pustules in the center (Weber *et al.*, 1966). Pustules vary from minute specks to large, irregular, mottled brown areas that arise when smaller lesion coalesce. In later stages, dried, broken remnants of pustules seen as small brown areas of necrosis are surrounded by narrow yellow haloes. The symptoms may develop on stems and pods of susceptible varieties. More recently, there has been research on development of resistant cultivars such as Sukhothai1 (ST1), Sukhothai2 (ST2), and Chiang-Mai60 (CM60) (Prathuangwong and Amnuaykit, 1989). Symptoms on resistant cultivars appear as occasional small chlorotic spots, but with no well-defined pustules (Hartwig and Lehman, 1951). They may also develop light green chlorosis and slight browning or may have fewer and smaller pustules (Groth and Braun, 1986).

The greatest outbreaks of bacterial pustule are encountered in the late rainy season from July to September. When favorable conditions of hot weather and periodic rain occur in Thailand, the disease is most severe when the plants are 30-40 days old. According to past investigations, the disease is widespread in soybean growing areas in the north, central, and northeast regions (Prathuangwong, 1983). During 1976, pustule disease was quite severe since the recommended varieties SJ1, SJ2, and SJ4 were highly susceptible to the disease.

X. axonopodis pv. *glycines* is a Gram-negative bacterium, rod shaped, does not form spores, bacterial cells have appendages for movement such as flagellum and pilus that are used for movement in liquid and on solid surfaces respectively (O'Toole *et al.,* 1999; Mattick, 2002). The movement of pathogenic bacteria on a host is important in disease progression such movement and attachment are an essential event for the initiation of infection. Limited to colonizing the water-conducting xylem vessels of plants, phytopathogenic bacteria develop biofilms that contribute to the blockage of sap flow resulting in plant stress and disease (Maxwell *et al.*, 2003; Meng *et al.*, 2005; Li *et al.*, 2007), 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 characteristics of *X. axonopodis* pv. *glycines* 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. Characterization of colonies on media such as nutrient glucose agar, beef fusion agar and wakimoto’s agar are yellow, smooth rimmed, production of yellow pigment and dissolvable in alcohol but not dissolve in water (Sincleair and Dhingra, 1975; Sincleair and Backmam, 1982).

4. Flagella

The benefits of flagella activity are not away easy to express in the same units as those of costs; that is specially the case for reproductive benefits, such as dispersal and sex (Table 1) (Macnab and De Rosier, 1988). The benefit in terms of the source acquisition and retention are more readily related to costs. Benefits in resource acquisition terms include the capacity to allow photolithotrophs to reach the ‘optimal’ position in the light and in the dark in opposing vertical gradients of light and nutrients in unmixed water bodies, and to allow phagothophs to maximize particle extraction from the medium for both swimming and attached flagellates as well allowing attached prototrophs to maximize inorganic C uptake. In terms of resource retention flagella mobility can help to avoid predation and avoid damage caused by high fluxes of photosynthetically active radiant and of UV-B. At the risk of being Panglossian, it appears to be possible to explain some at least of the occurrences of flagellates in various habitats, either as the dominant phase in the life-cycle or a temporally restricted phase in the life-cycle, in terms of cost-benefit analyses. However, agreement between cost-benefit prediction and what happens in the real world needs further investigation in terms of causal evolutionary terms (Silverman and Simon, 1974).

Flagella associated genes; swimming motility mediated by extracellular flagella that are responsible for motility and early interactions with hosts is an example of a motor functioning at the molecular level (Khater *et al*., 2007). The flagellum exports its own extracellular component proteins and assembles them at a distant tip and, together with its cognate sensory transduction system, receives sensory information from the cytoplasm which controls the direction of its high-speed rotation, a process critical for chemotaxis (Macnab, 1963; 2003; 2004). The filament of a flagellum is a tubular structure made up of 11 protofilaments, which are nearly longitudinal helical arrays of many hundred; 45-kDa flagellin molecules. The long helical filament of the flagellum is formed by about 30,000 subunits of flagellin (FliC) and is capped at its extremity by a hook-associated protein, FliD, which acts as a nucleation point for FliC monomers to polymerize into the growing filament (Aizawa, 1996). The filament is attached to a flexible hook (FlgE), extending from the cell surface by two hook-associated proteins FlgK and FlgL (Fahrner *et al*., 1994). Flagellum assembly is sequential: FlgK and FlgL rings must be present before FliD can polymerize, and the FliD cap must be in place before FliC can be incorporated

(Kubori *et al*., 1992). During assembly of the flagellum on the bacterial cell surface, structural proteins are exported via a flagellum specific export pathway and polymerize to form contiguous substructures (Moens and Vanderleyden, 1996; Journet *et al*., 2005). In addition to its role in motility, flagellin is a known elicitor of animal innate immune responses mediated by toll-like receptors (Pfund *et al.*, 2004). Flagellins are a PAMP that is recognizable by the innate immune systems of plants and animals (Gomez-Gomez and Boller, 2002; Gomez-Gomez, 2004; Nurnberger *et al*., 1994; 2004). Although most flagellin is assembled into flagella, flagellin can also leak into the bacterial environment during the construction of flagella (Komoriya *et al*., 1999), and flagellin is a component of the detritus associated with a bacterial colony. However, nonmotile pathogens can still caused disease symptoms, flagella motility is essential for the overall pathogenicity of bacterial plant pathogens (Haefele and Lindow, 1987; Panopoulos and Schroth, 1974).

4.1 Structure

The bacterial flagellum consists of several major elements (Figure 1). The passive (non-motor) structural elements are (i) the MS ring, rod, and L and P rings (collectively called the basal body), (ii) the hook and associated hook-filament junction zones, and (iii) the filament and its distal cap. The motor consists of (iv) a rotor/switch element or C ring, a peripheral membrane structure mounted on the cytoplasmic face of the MS ring, and (v) stator elements or Mot complexes that are embedded in the cell membrane and surround the MS and C rings. Finally, and central to the subject of this chapter, there is (vi) the type III flagella protein export apparatus, which consists in part of integral membrane components located in the center of the MS ring, and soluble or peripheral components such as the ATPase that drives the export process. Thus, everything beyond the MS ring has to be exported across the plane of the cell membrane, subunit by subunit, and delivered to its assembly destination at the tip of the growing structure. This is a logistical problem on a massive scale hundreds of thousands of protein subunits per cell per generation over large distances (up to 10 times the body length of the cell). Success requires not only the export of subunits but also their ordered assembly, without the aid of anything other than the exported proteins themselves (Macnab, 2004).

Table 1 Benefits of flagella

Benefits of flagella and restrictions on their applicability	**Alternatives to flagella in providing the benefits supplied by flagella and restrictions on their applicability**	**References**
1. Rotation of cells during mobility can help to constructively alternate the (limiting) photon flux density incident on different parts of the photosynthesis apparatus in phototrophs. Limited to vector radiation fields, organisms large enough to have a significant self shading effect.	Rotation occurs naturally (thermal motion or microturbulence) at "constructive" frequencies in cells less than a few µm in diameter, although such small cells may not have enough self-shading to benefit from this rotation.	Roven, 1994
2. Movement through a "homogenous" environment increases nutrient availability by decreasing diffusion boundary layer thickness (only significant for larger organisms) and hence increasing uptake of dissolved increasing from growth-rate-limiting concentrations for phototrophs and saprotrophs. More quantitatively important for phagotrophy by flagellates. Particles can contribute information (genes) as well as matter and energy**.**	Larger free-swimming animals rely on muscular activity.	Pahlow *et al.*, 1997

Table 1 (Continued)

Benefits of flagella and restrictions on their applicability	**Alternatives to flagella in providing the benefits supplied by flagella and restrictions on their applicability**	**References**
3. Movement of water over, or through, benthic organisms (e.g. sponged) aided by flagella activity	"Passive" flow due to currents, waves, relative to attached organisms with no flow generating	Vogel, 1981
4. Flagellates can maximize acquisition of resources from a spatially heterogenous environment, e.g. where resource gradients are in opposite directions in waters with little vertical water movement, photosynthestic flagellates can position themselves with near optimal light availability in daylight but deeper, in high nutrient concentrations, at night. Can work in free water bodies and in sediments/soils, but limited to where vertical water movement is slower than speed of swimming.	Muscle-based movements can also provide positioning in the water column which, for large organisms, can occur despite large vertical water movement. Muscle-based and pseudopodial (also actomyosin-based) movements can occur in sediments, as can gliding motility, all at velocities adequate to move through the opposing gradients to appropriate day and night positions. "Passive" movements related to buoyancy variations occur in cephalopods, teleosts, cyanobacteria and diatoms.	Raven and Richardson, 1984; Raven, 1997; Walsby, 1994
5. Flagellar ability may help to remove motile organisms from physiochemical or biotically damaging environments, although the flagellar apparatus is more	Other (e.g. actomyosin-based, "passive" buoyancy-related, gliding) can be involved in avoiding damaging environments. Chrysophyte	Raven, 1988; 1997

Table 1 (Continued)

Benefits of flagella and restrictions on their applicability	**Alternatives to flagella in providing the benefits supplied by flagella and restrictions on their applicability**	**References**
sensitive to UV-B than many cell functions, and movement from a damaging radiation environment using flagella depends on appropriate water movement regimes.	tricocysts (as offensive/defensive "ballistic missiles") are only effective over a few µm even with a muzzle velocity of 260m s^{-1}; "cruise missiles" would have a longer range.	Raven, 1994
6. Dispersal and sexual reproduction involves flagella in organisms which are flagellate throughout their life as well as many of those which spend most of their time as non-flagellate unicells or as multicellular organisms with few or no flagellate cells. Limitations as for resource acquisition and avoidance of damaging environments; only works in water or water films on terrestrial organisms, or in internal fertilization.	Passive (borne on water currents) fertilization and dispersal is an important adjunct to flagella motility of zoospores, flagellate sperm. Aquatic organisms with male gametes lacking flagella (red algae; pollen grains of seagrasses) can achieve submerged external fertilization. Ballistic' dispersal (fertilization) procedures much more effective in aerial than aquatic habitats, especially on a scale which is competitive with flagella activity.	

4.2 Chemical structure

Flagella are mostly composed of the protein flagellin, which is bound in long chains and wraps around itself in a left-handed helix. The number of protein monomers that it takes to make a single turn of the helix is determined by the protein subunits themselves.

The flagellum is attached to the cell through complex protein structures termed the hook and the basal body. One ring in the basal body rotates relative to the other causing the flagellum to rotate. The energy to drive the basal body is obtained from the proton motive force. In some fashion the translocation of protons from outside to inside the membrane causes the rotation of the flagellum. In a sense, the protons move through the wheel-like structure of the basal body (similar to a water wheel, except using protons) and this causes the rotation of the assembly including the flagellum. When *E. coli* is swimming through a solution the flagella turn counter-clockwise and push the microbe through solution. This behavior is termed smooth swimming. It is possible for *E. coli* to also reverse the direction of flagella rotation and when the flagella turn clockwise, they pull against the bacterial cell. Since *E. coli* is flagellated peritrichously (that is, at many positions), it is pulled in all directions and tumbles.

4.3 The flagellum as motor organelle

The bacterial flagellum is an organelle for cell propulsion. Although it carries the same name as the eukaryotic flagellum, the two organelles are in fact quite distinct. The bacterial flagellum is powered by proton-motive force (with contributions from the electrical potential $\Delta\Psi$ and the chemical potential ΔpH) or in some species sodium-motive force. The electrochemical energy is converted into torque, resulting in rotation of the flagella motor, which is attached via the MS ring to the basal body rod, to the hook, and then to an extremely long thin helical structure, the flagella filament (Figure 1). Rotation of this helical filament converts torque into thrust and propels the cell. The system contains a switch that can generate either counterclockwise or clock-wise rotation. The two directions have different consequences for the cell, forward motion and random reorientation. Chemical signals modulate the probabilities of the two rotational directions so as to produce the beneficial behavior called chemotaxis. For extensive reviews of motility and chemotaxis (Aizawa, 1996; Bourret and Stock, 2002; Stock and Surette, 1996; Macnab, 1996). A recent review on flagella assembly (Macnab, 2003) includes a more extensive treatment of flagella structure, motility and chemotaxis than that presented above.

4.4 The flagella gene system

Throughout this chapter, explicit mention of the names of genes and their products will largely be restricted to those that are central to the export process. The flagella / motility / chemotaxis gene system constitutes a regulon, i.e, an integrated hierarchy of controlled expression of about 50 genes, excluding receptor genes of which there are perhaps 20 (Kutsukake , 1994; Kutsukake *et al.*, 1990; Macnab, 1992). Regulation of gene expression plays a significant role in the processes of export and assembly in wild-type cells. At the top of the hierarchy (class 1) is the master operon, whose two genes must be expressed in order for any of the other genes in the regulon to be expressed. Class 2 operons contain primarily genes encoding flagella structural proteins, a few regulatory proteins, and components of the type III export pathway. Class 3 operons (actually classes 3a and 3b) contain genes encoding flagella structural proteins assembled late in the morphogenetic pathway (notably the filament protein, flagellin), the Mot proteins, and the chemotaxis proteins. The processes of genetic control and flagella protein export intersect directly at one point when a protein, FlgM, which inhibits transcription of late genes by sequestering a flagellum-specific sigma factor, is itself inactivated by expulsion from the cell by the type III apparatus surely one of the more unusual mechanisms of genetic regulation (Hughes *et al.,* 1993; Kutsukake, 1994). For are flagella assembly (Aldridge and Hughes, 2002).

4.5 The role of the capping proteins in assembly

In vivo the filament, the hook, and probably the rod employ a capping structure at the point of monomer assembly (the distal end). The best studied example is the filament. It was originally thought that the filament cap (made of FliD) functioned simply as a kinetic trap to give flagellin time to go from its pre-folded state (in which the terminal regions are in an unstable flickering a-helical state) to the state where it has undergone folding and quaternary interactions with its neighbors. However, a recent study indicates that the role of the cap, which has pentameric annular symmetry, is more akin to that of a chaperone, creating a chamber that actively assists the folding process and promotes the assembly by directing flagellin to its appropriate binding position (Yonekura *et al.*, 2002). It is likely that the same will hold true for the hook cap (FlgD) and rod cap (FlgJ). The rod cap has an interesting additional feature, namely, that its C-terminal domain has muramidase activity (Nambu and Kutsukake, 2002); mutants defective in this domain assemble as far as the rod and P ring (Hirano *et al.*, 2001). It is hypothesized that the muramidase domain is responsible for chewing a local hole in the peptidoglycan layer, thus permitting penetration by the rod and continuation of flagella assembly.

4.6 Flagella arrangement

Different species of bacteria have different numbers and arrangements of flagella (Figure 2) (Allen and Baumann, 1971; Atsumi *et al.*, 1992).

1. Monotrichous bacteria have a single flagellum (example *X. axonopodis* pv. *glycines*).
2. Lophotrichous bacteria have multiple flagella located at the same spot on the bacteria's surface which act in concert to drive the bacteria in a single direction (*Bartonella bacilliformis*).
3. Amphitrichous bacteria have a single flagellum each on two opposite ends (only one end's flagellum operates at a time, allowing the bacteria to reverse course rapidly by switching which flagellum is active) (example *Spirillum serpens*).
4. Peritrichous bacteria have flagella projecting in all directions (example *Erwinia carotovora* subsp. *carotovora*)

Some species of bacteria (those of spirochete body form) have a specialized type of flagellum called axial filament that is located in the periplasmic space, the rotation of which causes the entire bacterium to corkscrew through its usually viscous medium.

4.7 Components of the export apparatus

In describing the export apparatus, it is important to distinguish between (i) components that house the export apparatus but also function in the flagellum as a motor organelle, and (ii) components whose sole unction is in export. Included in the former category are structures like the MS and C rings, without which the export apparatus cannot assemble and function, but they do not in any direct sense function in export. The proteins that function directly in export or assembly are listed in Table 2, which also serves later for a comparison between type III flagella protein export and type III virulence factor secretion. There are nine proteins (FliH, FliI, FliJ, FlhA, FlhB, FliO, FliP, FliQ, and FliR) that are truly central to the flagella export apparatus, in the sense that they participate in the export of all known substrates (Minamino and Macnab, 1999). Three of the proteins-FliH, FliI and FliJ-are soluble. The remaining six are integral membrane proteins and are believed to be located in a patch of specialized membrane within a central pore in the basal-body MS ring (Macnab and Macneb, 1999; Macnab, 2003; Suzuki *et al.*, 1999). They range in size from 10 kDa (FliQ) to 75 kDa (FlhA), and vary in the predicted number of membrane spans from one (FliO) to eight (FlhA). Two of them (FlhA and FlhB) have large C-terminal cytosolic domains that interface with the soluble components. The evidence for association with the MS ring is still fragmentary. FliP and FliR have been found in the basal body Fan and Macneb, 1996; attempts to detect the others have been unsuccessful so far, perhaps because the detergent treatment used in preparation of basal bodies results in their dissociation. Genetic evidence (extragenic suppression) suggests an interaction between FlhA and the MS ring protein FliF (Kihara *et al.*, 2001). Also, given the physical pathway by which exported subunits reach their destination, the core of the MS ring seems the only logical location for the export apparatus. Although considerable effort has gone into studying the export components by biochemical and biophysical means, and many protein-protein interactions have been established, their detailed mechanistic roles are for the most part not well understood. The best understood are the ATPase FliI (Fan and Macnab, 1996), its regulator FliH (Minamino and Macneb, 2000; 2001; Minamino *et al.*, 2003), one of the membrane components, FlhB, which plays a role in determining export substrate specificity (Fraser *et al.*, 2003; Minamino and Macneb, 2000), and a putative general chaperone, FliJ (Fraser *et al.*, 2003; Minamino and Macneb, 2000). For the remaining components (FlhA, FliO, FliP, FliQ, and FliR), all that can be said for now is that they are essential for flagellar protein export and that FlhA appears to be important for association of the export complex with the MS ring (Kihara *et al.*, 2001) and the ATPase (Minamino *et al.*, 2003). In addition to the general components, there are at least three specific cytoplasmic chaperones, FlgN, FliS and FliT (Auvray *et al.*, 2001; Fraser *et al.*, 2003; Yokoseki *et al.*, 1995), which associate with their substrates, the hook-filament junction proteins, flagellin, and the filament capping protein, respectively. Whether or not they play a direct role in presenting their substrates to the export apparatus is not clear, but they do prevent substrate degradation (Ozin *et al.*, 2003). There is also a specific periplasmic chaperone, FlgA, for the P-ring protein (Nambu *et al.*, 1999). Finally, there are proteins that play important roles in various aspects of the assembly process, such as the hooklength control protein FliK (Hirano *et al.*, 2001; Patterson-Delafield *et al.*, 1973; Williums *et al.*, 1996), the muramidase / rodcapping protein FlgJ (Hirano *et al.*, 2001; Nambu *et al.*, 1999), the hook-capping protein FlgD (Ohnishi *et al.*, 1994), and the filament-capping protein FliD (Yonekura *et al.*, 2002). With the exception of FliD, these proteins are not present in the final flagella structure.

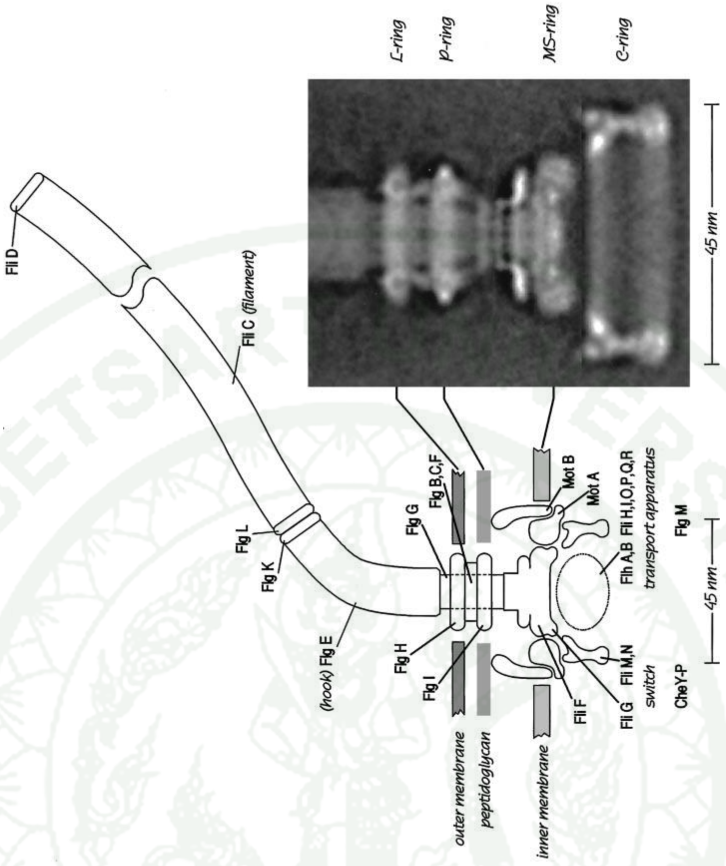

Figure 1 A schematic diagram of the flagella motor, drawn to scale, compared to arotationally averaged reconstruction of images of hook-basal bodies seen in an electron microscope. CheY-P is the chemotaxis signaling molecule that binds to FliM, and FlgM is the anti-sigma factor pumped out of the cell by the transport apparatus. The general morphological features are C-ring, MS-ring, P-ring, L-ring, hook, hook-associated proteins (which include the distal cap), and filament. MotA, MotB, and components of the transport apparatus (dashed ellipse) do not survive extraction with detergent and, therefore, are not shown on the right. This reconstruction is derived from rotationally averaged images of about 100 hook-basal body complexes of *Salmonella polyhook* strain SJW880 embedded in vitreous ice (Fan and Macnab, 1996).

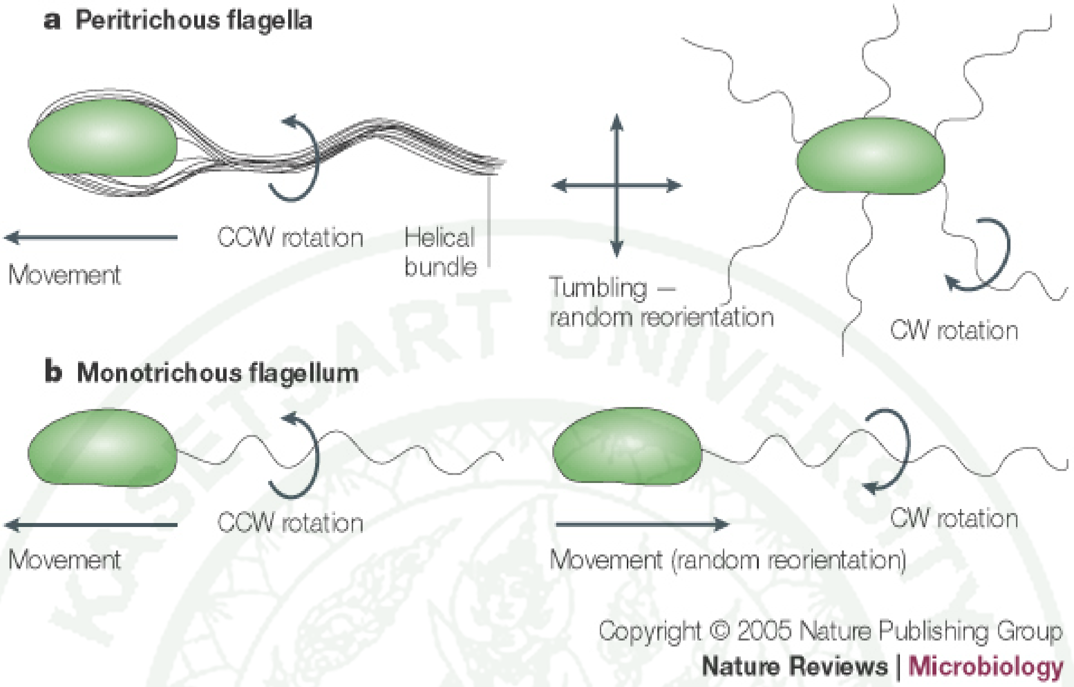

Figure 2 There are many schemes for flagellation in bacteria, of which peritrichous flagella and a single polar (monotrichous) flagellum are two types. A) In the case of peritrichous flagella, such as those found in *Escherichia coli*, counter-clockwise (CCW) flagella rotation results in the formation of a helical bundle that propels the cell forward in one direction in a smooth-swimming motion (a 'run'). By contrast, the presence of clockwise (CW) rotation causes unbundling of the helical bundle, allowing the bacterium to randomly reorient its direction (a 'tumble'). B) In the case of a single polar flagellum, CCW rotation propels the cell forward in a run, whereas CW rotation propels the cell backward with a concomitant random reorientation (Butler and Camilli, 2005).

4.8 Interactions among export components

Interactions among export components have been and continue to be an active subject of investigation. There are many such interactions, which probably occur in a dynamic fashion, at least as far as the soluble components are concerned (Minamino and Macneb, 2000; Zhu *et al.*, 2002). The ATPase FliI exists as a monomer in solution, or as a heterotrimer, FliI (FliH) 2, with its regulator FliH, which itself can exist as a homodimer (FliH) (Gonza´lez-Pedrajo *et al.*, 2002; Minamino and Macnab, 2000). The C-terminal domain of FliH interacts with the N-terminal domain of FliI; the catalytic C-terminal domain of FliI is not involved in the interaction. A short central region of FliH represents its dimerization domain. In the presence of ATP or analogs and also in the presence of phospholipids, FliI forms a hexamer (Claret *et al.*, 2003). We have already alluded to the fact that FliI interacts with FlhA and FlhB as judged by suppression studies (Minamino *et al.*, 2003) and biophysical measurements (Zhu *et al.*, 2002). FlhA and FlhB interact with themselves and with each other. The C-terminal region of FliJ, a general chaperone, interacts with FliH; FliJ also interacts with the cytosolic domain of the integral membrane protein FlhA. The association of FlhB with FliR, another membrane component, is strongly indicated by the existence of a natural FliR/FlhB fusion in Clostridium (Nolling *et al.*, 2001), and a similar inference regarding association of FliO and FliP may be made from the existence of a FliO/FliP fusion in *Buchnera* (van Ham *et al.*, 2003).

4.9 Comparison with type III secretion of virulence factors

Macnab (2003) have been report a comparison of type III export of flagella proteins and type III secretion of virulence factors. Where complete genomes are available, it appears that type III virulence systems only exist in flagellated Gram-negative species. In contrast, a number of nonpathogenic but flagellated species (e.g., *Caulobacter crescentus*), not surprisingly, lack type III virulence systems. The nonmotile, consistent with its life style. It does not seem to have a type III virulence system.

The important differences between the flagella and virulence systems are functional ones. In the case of the flagella system, with a few exceptions (the anti-j factor FlgM and the hook-length control protein FliK), the export substrates are incorporated into flagella structure, in some cases temporarily as scaffolds such as the hook cap but most often permanently. In the case of the virulence factor system, the early substrates are incorporated into bacterial structure (the needle complex), but the rest are either incorporated into host structure (e.g., the pore complex in the host plasma membrane) or as soluble factors that function either in the extracellular milieu or in some compartment of the host cell. A number of the structural components of the needle complex appear to be functionally homologous to those of the flagella hook-basal body complex, and in at least two cases (the flagella MS-ring protein FliF and the FliN component of its C ring) there is significant sequence similarity (Table 2). The role of FliN in motor rotation and switching is still rather mysterious. The existence of the needle complex that is known has no role other than in secretion and virulence and confirms the presumption that the hook-basal body complex is properly to be viewed as part of the flagella export apparatus as well as part of the flagellum as a motor organelle. The rod and L ring are also essential for total export to the external medium. When it comes to the components directly involved in the export and secretion processes themselves, the degree of sequence similarity is striking in almost all cases (Table 2). Interestingly, the probability values that this is a chance event can vary widely from species to species, and the similarity is not necessarily highest for a comparison within the same species. The case of FliH, where a comparison between FliH (*Treponema denticola*) and YscL (*Yersinia enterocolitica*) shows a far higher degree of similarity than between FliH (*Salmonella*) and YscL (*Y. enterocolitica*), and no match at all is found between FliH (*Salmonella*) and any *Salmonella* virulence factor secretion protein. Not surprisingly, there is no similarity between the virulence factors (effectors) and the exported flagella proteins, since they perform entirely different functions. It has been suggested that the type III virulence factor secretion system evolved from the type III flagella protein export system, since flagella are far more ancient (existing in very diverse genera) than the organisms that are targets for type III virulence systems (Macnab, 1996). However, it is possible that the original targets were other bacteria. Also, the possibility of lateral gene transfer cannot be ruled out. Finally, one could argue that evolution from a less complex structure (the needle complex) to a more complex (the flagellum) is more probable than the other way around. A comparison of the genomic organizations of the flagella export system and of the type III virulence systems reveals that the gene order of homologs is often preserved. As examples, we may cite *Chlamydia* lcrD-YscU (homologous to flhB-flhA) or *Salmonella* spaP-spaQ-spaR (homologous to flip-fliQ-fliR). This provides strong support (in addition to the amino acid similarities) for the idea that the two systems are evolutionarily related. There is a striking difference in the degree of clustering of the genes in the two systems. Although flagella export genes are clustered to varying degrees, virulence genes are very highly clustered, often to the point where all of the secretion apparatus genes (as well as many effector genes) exist as a single large cluster (often referred to as a pathogenicity island); for example, *Salmonella* pathogenicity island 1 (SPI-1) contains 30 genes. These islands are often flanked by direct repeats and the genes often have a higher GC content than the rest of the chromosome (Hueck, 1998).

5. Disease management of bacterial pustule

There are no resistant varieties for soybean grown for vegetable production. During 1976, the bacterial pustule outbreak was quite severe since the recommended varieties, namely SJ1, SJ2 and SJ4, were susceptible to the disease. The most widely used bactericides are copper-based, such as copper oxychloride and copper hydroxide. Copper is phytotoxic to some plants and should be used with caution. There is general concern caused by rapid development of resistant strains of bacterial pathogens. There is a considerable decrease in chemical applications and the need for sustainable agriculture has never been greater. Many of the chemicals used in plant disease control, however, are environmentally damaging. Since the chemical control methods require the application of toxic substances to plants, these chemicals find their way into human and animal food and pollute waterways, often causing pathology to fish, birds, and other wildlife.

Table 2 Type III flagella protein export components and a comparison of their homologs in virulence factor secretion systems (Macnab, 2004)

Flagella protein[a]	**Function/properties**	**Molecular mass (kDa)**	**Virulence protein**[a]	**Molecular mass (kDa)**
FliH	Regulator of FliI activity	25.8	n.d.[c]	-
		25.8	YscL (*Yersinia enterocolitica*)	24.9
FliH (*Treponema denticola*)		35.2	YscL (*Yersinia enterocolitica*)	24.9
FliI	Export ATPase	49.3	InvC	47.6
FliJ	General chaperone	17.3	n.d.[c]	-
FliK	Hook-length control	41.8	InvJ	36.4
FihB	Membrane component; interactions with soluble components; specificity switching	42.4	SpaS	40.1
FliO	Membrane component; functions unknown	13.1	n.d.[c]	-
FliP	Membrane component; cleaved signal sequence	26.8	YscR	24.1
FliQ	Membrane component; functions unknown	9.6	SpaQ	9.4
FliR	Membrane component; functions unknown	28.9	SpaR	28.5

Table 2 (Contained)

Flagella protein[a]	**Function/properties**	**Molecular mass (kDa)**	**Virulence protein**[a]	**Molecular mass (kDa)**
FliF	MS ring protein; necessary for export; interacts with FlhA	61.1	HrpC (*Pseudomonas syringae*); presumed lipoprotein	29.1
FlgE	Hook protein	42.1	PrgI	8.9
FlgJ	Muramidase and putative rod capping protein	34.4	n.a.[d]	-

[a] Unless otherwise stated, the proteins are from Salmonella, but they exist in essentially all flagellated species and type III virulence species.

[b] A parameter generated by BLASTP indicating the probability that the observed degree of sequence similarity would occur by chance.

[c] n.d., no homolog detected in *Salmonella*.

[d] n.a., not applicable; homology based on mutant phenotype or biochemical evidence; no

Additionally, many chemicals are toxic for only a limited range of pathogens, requiring the application of multiple chemicals in order to achieve broad protection. Chemicals may also have to be reapplied during a growing season if the chemicals are washed off the plants during a rain. A second method of controlling pathogens is through the use of disease resistant cultivars of plants. This method has not proven to be totally satisfactory because disease resistant cultivars may not produce the highest yield or highest quality crop compared to non-resistant ones. Also, because many pathogens exist as distinct strains, a cultivar which is resistant to one strain of a pathogen may not be resistant to a different strain. Recently, sustainable agriculture or organic farming systems with biological control have become more important. It is a strategy to decrease or replace chemical application. Research in Thailand is evaluating plant growth-promoting rhizobacteria (PGPR) and biological control agents primarily as new avenues of integrated disease management (IDM) in several crops. New approaches are needed for management of important diseases such as bacterial pustule of soybean (Prathuangwong and Kasem, 2004; Prathuangwong *et al*., 2005; 2008).

Application of biocontrol agents enhanced systemic resistance triggering in the plants is referred to as rhizobacterial mediated induced systemic resistance (ISR) that has been studied in several crop plants. ISR is activated by PGPRs through changing the physiological and biochemical reactions of the host leading to the synthesis of defense chemicals against the challenge pathogens. These defense substances in the PGPR treated plants may play either a direct or an indirect role in the suppression of pathogen development. The chemical substances involved phytoalexin, phenolic compound increase in the activities of PR protein, peroxidase increase in the levels of mRNAs encoding phenylalanine ammonia lyase (PAL) and enhanced lignification (Dorey *et al*., 1999; Ramamoorthy *et al*., 2002) have been reported in plants following treatment with PGPR strains.

6. Virulence, pathogenicity, and sigma factors

6.1 Virulence and pathogenicity factors

Virulence factors are molecules expressed and secreted by pathogens (bacteria, viruses, fungi and protozoa) that enable them to achieve the following: colonization of a niche in the host (this includes adhesion to cells) for example, trimeric autotransporter adhesins (TAA), immunoevasion, evasion of the host's immune response, immunosuppression, inhibition of the host's immune response, entry into and exit out of cells (if the pathogen is an intracellular one) and obtain nutrition from the host. Virulence factors are very often responsible for causing disease in the host because they are often responsible for converting non-pathogenic bacteria into dangerous pathogens. In bacteria, virulence factors are often encoded on mobile genetic elements, such as bacteriophages, and can easily be spread through horizontal gene transfer. Some bacteria, such as *E. coli* O157:H7, gain the majority of their virulence from mobile genetic elements. Strategies to target these specific virulence factors and mobile genetic elements have been proposed. Virulence factors are including; toxins, growth regulators, exopolysaccharide, suppressor, and enzymes (Choquer *et al.*, 2007; González-Lamothe *et al.*, 2009).

Shurtleff and Averre (Shurtleff and Averre, 1997) defined that pathogenicity is the ability of a pathogen to cause disease, whereas virulence is the degree of pathogenicity of a given pathogen. As the plant bacterial pathogens are extracellular, they deploy a delivery of secreted virulence factors to interfere with host cell processes from outside plant cells. These include production of protein virulence factors (effectors), which are directly injected into host plant cell cytoplasm via a specialized type III secretion path (Block and Alfano, 2011; Jin *et al.*, 2003; Lindeberg *et al.*, 2012), secretion of low molecular weight phytotoxins which are produced into apoplast (Bender *et al.*, 1999), production of exopolysaccharides (Denny *et al.*, 1995) and cell wall degrading enzymes (Boch and Bonas, 2001; Salmond, 1994). Bacteria evade, overcome or suppress antimicrobial plant defenses using these virulence factors, which elicit release of water and nutrients from host cells to colonize in the apoplast successfully.

Pathogenicity refers to the ability of an organism to cause disease (ie, harm the host). This ability represents a genetic component of the pathogen and the overt damage done to the host is a property of the host-pathogen interactions. Commensals and opportunistic pathogens lack this inherent ability to cause disease. However, disease is not an inevitable outcome of the host-pathogen interaction and, furthermore, pathogens can express a wide range of virulence. Virulence, a term often used interchangeably with pathogenicity, refers to the degree of pathology caused by the organism. The extent of the virulence is usually correlated with the ability of the pathogen to multiply within the host and may be affected by other factors (ie, conditional). In summary, an organism (species or strain) is defined as being pathogenic (or not), and depending upon conditions, may exhibit different levels of virulence (William *et al.*, 2002)

6.2 Pectate lyase as a virulence factor

Pectins are a major component of the plant cell wall and comprise one of the two major coextensive networks in which cellulose microfibrils are embedded (Carpita and Gibeaut, 1993). Pectic polysaccharides exist in the cell wall as either “smooth” regions of a linear copolymer of a-(1-4)-linked GalUA or “hairy” regions that have attached a-(1-2)-linked rhamnosyl residues that may be substituted with Ara- and Gal-rich side chains. The pectin structure is further elaborated by divalent cation cross-linkages and possible esterification to other cell wall polymers. Because of the contribution of both ionic and covalent linkages, the structure of pectin may be modified by the ionic strength of the apoplast, by enzymes that modify the charge of the GalUA residues, or by enzymes that cleave either the a-(1-4)-linked GalUA backbone or side chains of the hairy pectin regions. Because plant cells undergo dramatic changes in shape and developmentally regulated episodes of cell separation, in which the pectin network is systematically disassembled, pectin metabolism is critical to many developmental processes.

A wide range of enzymes are known to catalyze aspects of pectin modification and disassembly. The best characterized are *exo*- and *endo*-PGs, pectate lyase, pectin methylesterase, and b-galactosidase, which has been proposed to have the capacity to reduce the apparent molecular size of pectic polymers by cleaving neutral side chain residues (De Veau *et al.*, 1993). In addition, it is likely that there are as-yet-undiscovered enzymes that may play critically important roles in cleaving covalent cross-linkages that tether pectins to other structural networks within the cell wall. Because of the extensive study of polygalacturonase-mediated pectin disassembly, this review summarizes what is known about the complexity and structure of genes encoding plant polygalacturonase s and their role in developmental processes.

Pectate lyases, otherwise known as pectate transeliminases, catalyse the eliminative cleavage of de-esterified pectin, which is a major component of the primary cell walls of many higher plants (Carpita and Gibeaut, 1993; Zhihua *et al*., 2011). Pectate lyases have been found in bacteria (Lei *et al.*, 1987; Nasser *et al.*, 1993), fungi (Debing *et al.*, 2006), and plant (Zhao *et al.*, 2008).

Pels are extracellular enzymes secreted by pathogenic organisms affecting plants. The enzymes degrade the pectate component of the middle lamella and cell wall of higher plants and have been shown to be causally involved in soft rot diseases (Lietzke *et al.*, 1994). The soft rot *Erwinia* spp., which are Gram-negative bacteria, secrete several types of pectic enzymes of which the most important for virulence are the Pels. *Erwinia* spp. typically produces multiple isozymes of independently regulated extracellular Pels. The extracellular isozymes belong to two gene families, pelADE and pelBC. There is 50 to 85% amino acid sequence identity within a family and 22% or lower identity between the families (Hinton *et al.*, 1989; Tamaki *et al.*, 1988). The pelADE family contains one disulfide bond and the pelBC family contains two. The *Erwinia* Pels also share regions of sequence similarity with fungal pectin lyases and pollen and style plant proteins (Wing *et al.*, 1989; Rafnar *et al.*, 1991). A third gene family of intracellular Pels is also produced in some pathogenic species but is unrelated in sequence to the extracellular families (Hinton *et al.*, 1989). Pels are exported through the inner membrane by the secretory-dependent pathway and the extracellular forms are transported across the outer membrane by products of the out gene family (He *et al.*, 1991; Condemine *et al.*, 1992; Lindeberg and Collmer, 1992).

Pels cleave a-1,4-linked galacturonic acid residues of the pectate component of the plant cell wall by a β-elimination mechanism that generates an unsaturated galacturonosyl residue at the nonreducing end (Collmer and Keen, 1986). The *Erwinia chysanthemi* enzymes act preferentially on unmethylated pectin, cleaving internal glycosidic linkages relatively infrequently and then exolytically cleaving exposed termini more frequently (Preston *et al.*, 1992). The different Pel isozymes catalyze the cleavage reaction at pH optima ranging from 8 to 11 (Kaewnum *et al.*, 2005), yielding primarily either dimer (pelADE family) or trimer (pelBC family) end products. In addition to mechanistic differences, the Pel enzymes differ considerably in their host range and relative maceration properties, with PelE considered to be the most virulent of the *E. chysanthemi* EC16 Pel enzymes (Tamaki *et al*., 1988).

Calcium is required for in vitro activity of all Pels, but it has not been established whether calcium binds to the enzyme or simply cross-links the strands of the substrate, PGA, into a structure that is recognized by Pels (Crawford and Kolattukudy, 1987).

Kaewnum *et al.* (2005) have been reported a transposon insertional mutant (KU-P-M670) of *X. axonopodis* pv. *glycines* derived from wild-type strain KU-P-34017 lost the ability to induce the hypersensitive response (HR) on tobacco and pepper but retained its HR induction capacity on cucumber, sesame, and tomato. The mutation also resulted in loss of ability to cause a potato soft rot and express pectolytic activity at pH 6.5. An approximate 1.4-kb DNA fragment carrying the transposon insertion contained a single open reading frame that showed high homology with PSTRU-3, a pectate lyase gene in *X. axonopodis* pv. *malvacearum*. Complemented KU-P-M670 regained HR induction on tobacco and also pectolytic activity. Treatment of plants with inhibitors of eukaryotic metabolism blocked HR induction by wild-type strains and by complemented KU-P-M670. The presence of the pectate lyase homolog, which we designated *xagP*, in 26 *X. axonopodis* pv. glycines strains was highly correlated with their ability to induce an HR on tobacco. To our knowledge, this is the first study indicating a role for a functional pectate lyase in induction of a plant HR.

6.3 Sigma factors

Sigma factors are a class of proteins constituting essential dissociable subunits of prokaryotic RNA polymerase. RNA polymerase holoenzyme complex consists of core RNA polymerase and a sigma factor executes transcription of a DNA template strand. Once initiation of RNA transcription is complete, the sigma factor can leave the complex (Gruber and Gross, 2003; Sharma and Chatterji, 2010). The association of appropriate alternative sigma factors with core RNA polymerase provides a mechanism for cellular responses mediated through redirection of transcription initiation. Sigma factors provide promoter recognition specificity to the polymerase and contribute to DNA strand separation; they then dissociate from RNA polymerase core enzyme following transcription initiation (Borukhov and Nudler, 2003). As the regulon of a single sigma factor can be comprised of hundreds of genes, sigma factors provide effective mechanisms for simultaneously regulating large numbers of prokaryotic genes. In some cases, the genes comprising a sigma factor regulon have a clearly defined primary function such genes regulated by the sporulation sigma factors in *Bacillus subtilis* (Piggot and Hilber, 2004); in others, the genes comprising a regulon contribute to multiple functions as the stationary-phase and general stress response genes regulated by *Listeria monocytogenes* (Kazmierczak *et al.,* 2003). One newly emerging field is identification of the specific roles of alternative sigma factors in regulating expression of virulence genes and virulence-associated genes in bacterial pathogens.

Virulence and virulence-associated genes are those that contribute to at least one aspect of bacterial disease transmission and infection processes. Specifically, virulence genes encode proteins whose functions are essential for the bacterium to effectively establish an infection in a host organism. Examples of virulence genes are *L. monocytogenes inlA*, which encodes the internalin-A protein important for invasion of nonprofessional phagocytes (Lingnau *et al.,* 1995), and the *spv* gene cluster of *Salmonella enterica*, which allows for bacterial growth inside acrophages (Libby *et al.,* 2000). In contrast, virulence-associated genes can contribute to bacterial survival in the environment such the *ica* operon of *Staphylococcus aureus*, which produces an adhesin important for biofilm formation on plastic surfaces such as those on indwelling medical devices (Mc Kenney *et al.*, 1998) or to survival in the host such as *bsh* of *L. monocytogenes,* encoding bile salt hydrolase, which enhances bacterial survival in the intestinal environment prior to intracellular infection (Dussurget *et al.,* 2002). Therefore, activation of virulence-associated genes may enhance the capacity of the bacterium to spread to new individuals or to survive passage through a host organism. As alternative sigma factors have been shown to regulate expression of both virulence and virulence-associated genes, these sigma factors can contribute both directly and indirectly to bacterial virulence (Kazmierczak *et al*., 2005).

A sigma factor is a protein needed only for initiation of RNA synthesis (Gruber and Gross, 2003). It is a bacterial transcription initiation factor that enables specific binding of RNA polymerase to gene promoters. The specific sigma factor used to initiate transcription of a given gene will vary, depending on the gene and on the environmental signals needed to initiate transcription of that gene. Every molecule of RNA polymerase holoenzyme contains exactly one sigma factor subunit, which in the model bacterium *E. coli* is one of the listed. The number of sigma factors varies between bacterial species (Gruber and Gross, 2003; Sharma and Chatterji, 2010). *E. coli* has seven sigma factors. Sigma factors are distinguished by their characteristic molecular weights. For example, σ^{70} refers to the sigma factor with a molecular weight of 70 kDa (Gruber and Gross, 2003).

Sigma factors can be classified into two structurally unrelated families: the -70 and the -54 families. For certain sigma factors, we use the predominant designation from the literature instead (e.g., FliA). The -70 family includes primary sigma factors (e.g., *Bacillus subtilis* -A) as well as related alternative sigma factors (Missiakas and Raina, 1998; Paget and Helmann, 2003). Alternative sigma factors within the -70 family are further categorized by the physiological processes they control, e.g., stress response. In general, these groupings by function also correlate with phylogenetic relationships among the protein sequences (Paget and Helmann, 2003). Within the -70 family of sigma factors is a large, phylogenetically distinct subfamily called the extracytoplasmic function (ECF) factors. These sigma factors are responsible for regulating a wide range of functions, all involved in sensing and reacting to conditions in the membrane, periplasm, or extracellular environment (70). Structurally, -70 family factors have four major regions, with the highest levels of conservation in regions 2 and 4. Sub-regions within region 2 are involved in promoter melting (region 2.3) and -10 sequence recognition (region 2.4). Region 4.2 is involved in -35 recognition. For a recent review on the -70 family of sigma factors (Paget and Helmann, 2003).

Although no sequence conservation exists between -54 and -70-like family members, both types bind to core RNA polymerase. However, the holoenzyme formed with -54 sigma factors has different properties than the -70 holoenzyme. While the C terminus (region III) of -54 enables DNA binding, all -54 species require a separate activator protein along with the core RNA polymerase (RNAP) to form an open promoter complex. The -54 N terminus, which inhibits isomerization in the absence of the appropriate activator, stimulates initiation upon activation (Buck *et al.*, 2000). Further, promoter structures recognized by -54-RNAP differ from those recognized by -70-RNAP. -54 promoters are highly conserved, short sequences that are located at positions -24 and-12 upstream of the transcription initiation site, whereas -70 promoter sites are typically located at -35 and -10 upstream. -54 promoters, which are called -24/-12 promoters, are almost completely invariant at the -24/-12 positions (GG and GC, respectively) and in their spacing in both gram-negative and gram-positive bacteria. For reviews on the structure-function relationships of -54 (Buck *et al.*, 2000; Merrick, 1993). Kazmierczak *et al.* (2005) presented several examples of alternative sigma factors that have been shown to contribute to virulence in at least one organism. The text is organized by sigma factor to include the three subfamilies (stress response, -28, and ECF) within the -70 family, as well as those within the -54 family. For each sigma factor, when applicable, examples will be drawn from multiple bacterial species (Kazmierczak *et al*. 2005).

rpoE is the first gene of an operon that also contains *rseA*, *rseB*, and *rseC* (regulator of sigma E, genes A, B, and C) (de las Penas *et al.*, 1997; Missiakas *et al.*, 1997). Sigma E is a minor sigma factor, specializing in responses to the effects of heat shock and other stresses on membrane and periplasmic proteins. It is regulated by a multistep protease system that sense disruptions in those proteins. Its transcriptional initiation targets include another minor sigma factor, Sigma 32 (Milton and Saier, 1998). σ^E is an alternative sigma factor that controls the extracytoplasmic stress response in *E. coli*. σ^E is essential at high temperatures but was previously thought to be nonessential at temperatures below 37°C. De Las Penas *et al.* (1997) presented evidence that σ^E is an essential sigma factor at all temperatures. Cells lacking σ^E are able to grow at low temperatures because of the presence of a frequently arising, unlinked suppressor mutation. For the sigma N or *rpoN* gene Kohler *et al.* (1989) reported the RpoN protein was originally identified in *E. coli* as a sigma (σ) factor essential for the expression of nitrogen regulons. In the present study we cloned the *Pseudomonas putida rpoN* gene and identified its gene product as a protein with an apparent molecular weight of 78,000. The RpoN protein, which was originally discovered as an indispensable factor for the expression of the *ntr* (nitrogen regulation) and *nif* (nitrogen fixation) metabolic operons (Hirschman *et al.*, 1985), recognizes promoter sequences containing invariant GG and GC sequences located 24 and 12 nucleotides, respectively, upstream of the transcription start point (Dixon, 1986). A mutant *rpoN* gene was constructed by in vitro insertion mutagenesis with a kanamycin cassette. A *P. pufida rpoN* mutant was then isolated by replacement of the intact chromosomal *rpoN* gene by the mutant *rpoN* gene through homologous recombination. Examination of the phenotypes of the *P. putida rpoN* mutant thus obtained allowed the identification of a series of metabolic functions whose expression depends upon the RpoN factor. The *rpoN* mutation in *P. putida* affected the utilization by this organism of nitrate, urea, and uncharged amino acids, namely, alanine, glycine, isoleucine, leucine, and serine, as nitrogen sources. The mutation also affected the utilization of the above-mentioned amino acids, as well as lysine, C4-dicarboxylates (succinate, fumarate), and a-ketoglutarate, as carbon sources. In contrast to the *P. putida* wild-type strain, the *rpoN* mutant was nonmotile. The colony morphology of the mutant strain was different from that of the wild-type strain. Studies on the expression of the TOL plasmid catabolic operons in the mutant strain demonstrated that transcription from the upper-operon promoter and from the *xylS* gene promoter requires the RpoN σ factor (Kohler *et al.*, 1989).

MATERIALS AND METHODS

1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 3. Strain of *X. axonopodis* pv. *glycines* 12-2 was grown on nutrient glucose agar (NGA) at 28°C overnight (Kaewnum *et al.*, 2006), which was composed of: 5.0 g/L bacto-peptone (Difco™, Becton, Dickinson Co., Sparks, Maryland), 3.0 g/L beef extract (Difco™), and 2.5 g/L glucose. *Escherichia coli* strains were grown on Luria Bertani (LB) agar or broth (10.0 g/L bacto-typtone, 5.0 g/L yeast extract (Difco™), 10.0 g/L NaCl and 15.0 g/L agar (pH 7.0) at 37 ^{o}C. *X. axonopodis* pv. *glycines* strain 12-2 was revived from stock culture of Department of Plant Pathology, Faculty of Agriculture, Kasetsart University and streaked on NGA. A single yellow-pigmented colony was purified by streaking onto NGA for Koch's postulate test on soybean susceptible cultivar (cv. SJ4). The culture was stored in a 20% glycerol solution, kept at -20 ^{o}C, and slant cultures were kept at 4 ^{o}C for routine use. Media were amended with appropriate antibiotics at the following concentration: ampicillin (50 µg/mL) and kanamycin (50 µg/mL), gentamicin (25 µg/mL), or spectinomicin (50 µg/mL) for *X. axonopodis* pv. *glycines* mutants, *E. coli* carrying plasmid vector (Table 3).

Table 3 Bacterial strains and plasmids

Bacterial strain or plasmid	**Relevant characteristic [1/]**	**Reference or source**
Escherichia coli		
TOP10	lacZΔM15, endA1, recA1, hsdR, mcrA	Invitrogen
JM109	recA1, endA1, gyrA96, thi-1, hsdR17 (rK-mk+), e14– (mcrA–), supE44, relA1, Δ(lac-proAB)/F’[traD36, proAB+, lac Iq, lacZΔM15]	Promega
TOP10 (pUC19-FLGC)	*E. coli* TOP10 carrying pUC19-FLGC	This study
TOP10 (pUC19-FLIC)	*E. coli* TOP10 carrying pUC19-FLIC	This study
TOP10 (pUC19-FLID)	*E. coli* TOP10 carrying pUC19-FLID	This study
TOP10 (pUC19-RPOE)	*E. coli* TOP10 carrying pUC19- RPOE	This study
TOP10 (pUC19-RPON)	*E. coli* TOP10 carrying pUC19- RPON	This study
TOP10 (pBBR-FLGC)	*E. coli* TOP10 carrying pBBR-FLGC	This study
TOP10 (pBBR-FLIC)	*E. coli* TOP10 carrying pBBR-FLIC	This study
TOP10 (pBBR-FLID)	*E. coli* TOP10 carrying pBBR-FLID	This study
TOP10 (pBBR-FLID)	*E. coli* TOP10 carrying pBBR- RPOE	This study
TOP10 (pBBR-FLID)	*E. coli* TOP10 carrying pBBR- RPON	This study
X. axonopodis* pv. *glycines		
12-2	Virulence strain	Kaewnum *et al.,* 2005
12-2 *flgC*:: Km^{r}	*flgC*::Km^{r} of 12-2 by gene deletion	This study
12-2 *fliC*::Gm^{r}	*fliC*::Gm^{r} of 12-2 by gene deletion	This study
12-2 *fliD*::Km^{r}	*fliD*::Km^{r} of 12-2 by gene deletion	This study

Table 3 (Continued)

Bacterial strain or plasmid	**Relevant characteristic $^{1/}$**	**Reference or source**
12-2 *rpoE*::Km^r	*rpoE*::Km^r of 12-2 by gene deletion	This study
12-2 *rpoN*::Km^r	*rpoN*::Km^r of 12-2 by gene deletion	This study
flgC::Km^r (pBBR-FLGC)	*fliC*::Km^r of 12-2 carrying pBBR-FLGC	This study
fliC::Gm^r (pPZP201-FLIC)	*fliC*::Gm^r of 12-2 carrying pPZP201-FLIC	This study
fliD::Km^r (pBBR-FLID)	*fliD*::Km^r of 12-2 carrying pBBR-FLID	This study
fliD::Km^r (pBBR-RPOE)	*fliD*::Km^r of 12-2 carrying pBBR-RPOE	This study
fliD::Km^r (pBBR-RPON)	*fliD*::Km^r of 12-2 carrying pBBR-RPON	This study
12-2 *xagP*::Gm^r	*xagP::*Gm^r of 12-2 by gene deletion	This study
12-2 *xagP*::Gm^r (pPZP201-XAGP)	*xagP*::Gm^r of 12-2 carrying pPZP201-XAGP	This study
12-2 *fliD&xagP* ::Km^{r}Gm^r	*fliD&xagP*::Km^r Gm^r of 12-2 by gene deletion	This study
12-2 *fliC&fliD*:: Gm^{r}Km^r	*fliC&fliD*::Gm^r Km^r of 12-2 by gene deletion	This study
12-2 *fliD&fliC*:: Km^{r}Gm^r	*fliD&fliC*::Km^r Gm^r of 12-2 by gene deletion	This study
fliD&xagP::Km^{r}Gm^r (pPZP201-FLID&XAGP)	*fliD&xagP*::Km^r Gm^r of 12-2 carrying pPZP201- FLID&XAGP	This study
fliC&fliD::Gm^{r}Km^r (pPZP201-FLIC&FLID)	*fliC&fliD*::Gm^r Km^r of 12-2 carrying pPZP201-FLIC&FLID	This study
fliD&fliC::Km^{r}Gm^r (pPZP201-FLID&FLIC)	*fliD&fliC*::Km^r Gm^r of 12-2 carrying pPZP201-FLID&FLIC	This study

Table 3 (Continued)

Bacterial strain or plasmid	Relevant characteristic [1/]	Reference or source
Plasmid		
pUC19	ColE1; lacZ, Amp^r	Promega
pBBR1MCS-5	Broad host range cloning vector, lacZ and Gm^r	Kovach *et al.*, 1995
pPUC19-FLGC	408-bp *flgC* fragment from 12-2 carrying kanamycin insertion cloned in pUC19, Km^r and Amp^r	This study
pUC19-FLIC	1.4-kb *fliC* fragment from 12-2 carrying gentamicin insertion cloned in pUC19, Gm^r and Amp^r	This study
pUC19-FLID	1.5-kb *fliD* fragment from 12-2 carrying kanamycin insertion cloned in pUC19, Km^r and Amp^r	This study
pUC19-XAGP	1.4-kb *xagP* fragment from 12-2 carrying gentamicin insertion cloned in pUC19, Gm^r and Am^r	This study
pUC19-RPOE	582-kb *rpoE* fragment from 12-2 carrying gentamicin insertion cloned in pUC19, Gm^r and Am^r	This study
pUC19-RPON	820-kb *rpoN* fragment from 12-2 carrying gentamicin insertion cloned in pUC19, Gm^r and Am^r	This study
pBBR-FLGC	408-pb sequence of *flgC* in the pBBR1MCS-5, Km^r and Gm^r	This study
pBBR-FLIC	1,497-pb sequence of *fliC* in the pBBR1MCS-5, Km^r and Gm^r	This study

Table 3 (Continued)

Bacterial strain or plasmid	**Relevant characteristic** [1/]	**Reference or source**
pBBR-FLID	1,559-bp sequence of *fliD* in the pBBR1MCS-5, Km^r and Gm^r	This study
pPZP201-XAGP	1400-bp sequence of *xagP* in the pPZP201, $Spec^r$	This study

[1/] Am^r= ampicillin resistance, Gm^r= gentamicin resistance, Km^r=kanamycin resistance, $Spec^r$=spectinomycin resistance.

2. Identification *X. axonopodis* pv. *glycines* pathogenicity associated genes, *xagP, flgC, fliC,* and *fliD*

The pectase lyase; flagella hook protein; flagella protein; and flagellin and hook associated protein 2 encoded by *xagP; flgC; fliC;* and *fliD* of *X. axonopodis* pv. *glycines* strain 12-2 respectively, *XagP* has been reported to play a role in activating HR induction, where *fliC* and *fliD* functions as a flagella filament and capping structure at the distal end of the filament essential for motility. Bacterial flagella (*flgC, fliC* and *fliD*) and pectase lyase (*xagP*) genes associated with flagella biosynthesis that controlled movement, cell attachment, and biofilm formation are thought to be important factors in pathogen virulence. Then, the association of specific genes with motility and biofilm formation in motile bacteria strain 12-2 was determined. Although these gene have been shown to play significant roles in pathogenesis of certain bacterial pathogens.

2.1 Cloning and construction

A targeted single gene mutation in *flgC; fliC; fliD;* and *xagP* in *X. axonopodis* pv. *glycines* strain 12-2 was accomplished via gene deletion (Ichinose *et al.,* 2003b). The flanking fragments of targeted gene were amplified from strain 12-2 using primers flgC-A and flgC-*AscI*-B, flgC-*AscI*-C and flgC-D; fliC-A and fliC-*AscI*-B, fliC-*AscI*-C and fliC-D; fliD-A and fliD-*AscI*-B, fliD-*AscI*-C and fliD-D;

xagP-A and xagP-*AscI*-B, xagP-*AscI*-C and xagP-D (Table 4) that are based on the flanking sequence of *flgC, fliC, fliD;* and *xagP* have been previously identified in *X. axonopodis* pv. *citri* the accession no. NC_003919; and no. AY725200, respectively. The fragments containing the region of up and downstream of *flgC, fliC, fliD,* and *xagP* were ligated into pUC19 (Quick Ligation^{TM} Kit, New England Biolabs). Briefly, 7 µL pUC19 containing the region of up and downstream of each individual gene mixed with 3 µL kanamycin gene fragment for *flgC* and *fliD* mutation and added 3 µL gentamicin gene fragment for *fliC* and *xagP* mutation and added 10 µL T4DNA ligation mixed, incubated at 16^{o}C overnight; the mixture was transformed by electroporation into *E. coli* Top10 (Invitrogen, CA, USA) as previously described by Athinuwat *et al.* (2009). Briefly, 2 µL of individual ligation (pUC19-FLGC, pUC19-FLIC, pUC19-FLID, and pUC19-XAGP) added into 50 µL of *E. coli* Top10 electrocompetent cells and subjected to electroporation in a 0.1 cm cuvette at 1.8 kV, 25 µF, and 200 Ω. Electroporated cells were added SOC medium broth and incubated at 37°C for 1 h.

Clones were selected on LB agar amended with kanamycin (50 µg/mL) or gentamicin (25 µg/mL) for *flgC* and *fliD* clones or *fliC* and *xagP* clones, respectively. The target sequences were shown by digestion enzyme and separation by gel electrophoresis. Confirmation of the plasmid constructs, pUC19-FLGC, pUC19-FLID; pUC19-FLIC, pUC19-XAGP were done by PCR and sequencing. The carrying kanamycin and gentamicin resistant genes were identified by PCR with primers flgC-A and flgC-D, fliD-A and fliD-D; fliC-A and fliC-D, xagP-A and xagP-D (Table 4), respectively.

2.2 Mutagenesis

Transformation pUC19-FLGC, pUC19-FLID; pUC19-FLIC, pUC19-XAGP into strain 12-2 was performed by electroporation as previously described by Athinuwat *et al.* (2009). Briefly, 2 µL of individual construct (pUC19-FLGC, pUC19-FLIC, pUC19-FLID, and pUC19-XAGP) added into 80 µL of strain 12-2 electrocompetent cells and subjected to electroporation in a 0.25 cm cuvette at 2.5 kV, 25 µF, and 200 Ω. Electroporated cells were added to fresh nutrient broth yeast extract (NBY) broth and incubated at 28°C for 2 h. Transformants were selected on NBY agar containing 50 µg/mL of kanamycin for selected *flgC* and *fliD* mutants and 25 µg/mL of gentamicin for *fliC* and *xagP* mutants. Resistant clones were subsequently verified for kanamycin or gentamicin genes insertion by PCR. The mutants were screened for ability of swimming motility, pathogenicity, and HR induction host and nonhost.

2.3 Sequencing and evaluation

The sequences of resistant clones were identified by PCR amplification with primers; flgC-A and flgC-D, fliC-A and fliC-D, fliD-A and fliD-D, xagP-A and xagP-D (Table 4) of *flgC, fliC, fliD,* and *xagP* mutants respectively. Analysis of sequences was conducted using BLAST and the DNASTAR Lasergene software package (DNASTAR, Wl, USA). The MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTALW. MegAlignment was then calculated for the *flgC, fliC, fliD,* and *xagP* sequences as available from GenBank for selected *Xanthomonas* spp.

2.4 Double mutation

Double mutation in *fliC&fliD, fliD&fliC,* and *xagP&fliD* were investigated. Transformation pUC19-FLID into 12-2 *fliC*::Gen^r, pUC19-FLIC into 12-2 *fliD*::Km^r, pUC19-XAGP into 12-2 *fliD*::Km^r was performed by electroporation as described above. Transformants were selected on NBY agar containing appropriate kanamycin 50 µg/mL and gentamicin 25 µg/mL. Individual colonies of putative mutants were screened for swimming motility and their pathogenicity on soybean.

2.5 Complementation

The amplified fragment 408; 1,497; 1,559; 1,400; 582 and 820bp sequence comprising *flgC; fliC; fliD; xagP; rpoE* and *rpoN* in pUC19 were digested with *EcoRI*; *SacI* and *BamHI; BamHI* and *HinDIII; SacI* and *BamHI* respectively and ligated into the multiple cloning site vector pBBR1MCS-5 (Kovach *et al.*, 1995) (pBBR-FLGC, pBBR-FLID, pBBR-RPOE, and pBBR-RPON) and pPZP201 (pPZP-FLIC, pPZP-XAGP, pPZP-FLIC&FlID, pPZP-FLIC&FLID, and pPZP-FLID&XAGP). The individual construct mobilized into mutant strains *flgC*, *fliC, fliD, xagP*, *rpoE*, and *rpoN* and double mutant including *fliC&fliD, fliD& fliC*, *fliD&xagP* respectively by electroporation according to the method described above. Selection was made on NBY agar amended with kanamycin (50 mg/mL) and gentamicin (25 mg/mL) for *flgC*, *fliD*, *rpoE*, and *rpoN* whereas *fliC, xagP* and double mutants *fliC&fliD, fliD& fliC,* and *fliD&xagP* were selected on NBY amended with gentamicin (25 mg/mL) and spectinomicin (100 mg/mL). They were verified by PCR and by their motility and pustule disease induction on soybean cv. Spencer and nonhost.

Table 4 Polymerase chain reaction primers

Primer	Sequence	Description
flgC-A	CAT CCC AGT CAA ACG ACA CCT	Amplification of 481-bp of upstream *flgC*
flgC-*AscI*-B	TTC GGC GCG CCG AAT ACC AAC GGA AGA CTC CAC ATG A	
flgC-*AscI*-C	TTC GGC GCG CCG AAT TGA CTT ATT GGC CCG TGA TCG	Amplification of 454-bp of downstream *flgC*
flgC-D	TGG CAC AAA CAT TGC TTC CTC C	
fliC-A	5’ AAC GAG ACC ACT TCC ACC GA 3’	Amplification of 559-bp of upstream *fliC*
fliC-*AscI*-B	5’ TTC GGC GCG CCG AAT TGG ACG GAT TGT TGA CGC G 3’	
fliC-*AscI*-C	5’ TTC GGC GCG CCG AAC TCC TAA GCG ATT TTT CCG GCA 3’	Amplification of 500-bp of downstream *fliC*
fliC-D	5’GCC AAC TCA TTG CTCGAATCC3’	
fliD-A	5’ AAT CCC AGC GCT TCG GTC AA 3’	Amplification of 500-bp of upstream *fliD*
fliD-*AscI*-B	5’ TTC GGC GCG CCG AAT CCT GTC AGG GTA TTG CCG AT 3’	

Table 4 (Continued)

Primer	**Sequence**	**Description**
fliD-*AscI*-C	5’ TTC GGC GCG CCG AAG TTG ATT CTC GTC TGG G 3’	Amplification of 500-bp of downstream *fliD*
fliD-D	5’ GCC CTG GAA ATC GTC GAC AA 3’	
xagP-*BamHI*-A	5’ ATGGATCC CAG CCT TGG ACG GAT TGT TGA 3’	Amplification of 500-bp of upstream *xagP*
xagP-*HinDIII*-B	5’ TAA AGCTT GAT AGG CCT TAC TTG TAT CGG C 3’	
xagP-*BamHI*-C	5’ ATGGATCC CAA CGC TTA CGT ATT TCG TC 3’	Amplification of 500-bp of downstream *xagP*
xagP-*SacI*-D	5’ TAGAGCTC GTC AAC AAT CCG TCC AAG GC 3’	
fliC-C-*SacI*-F	ATGGATCC CAA CGC TTA CGT ATT TCG TC 3’	Amplification of *fliC* (1497-bp)
fliC-C-*BamHI*-R	5’ TAGAGCTC GTC AAC AAT CCG TCC AAG GC 3’	
fliD-C-*BamHI*-F	5’ ATGGATCC CAG CCT TGG ACG GAT TGT TGA 3’	Amplification of *fliD* (1559-bp)
fliD-C-*HinDIII*-R	5’ TAA AGCTT GAT AGG CCT TAC TTG TAT CGG C 3’	
rpoE-F	5’ ATG ACT GAC ACC GAT ACC GTG 3’	Amplification of 582-bp of *rpoE*
rpoE -R	TTG CAT GGT CTC CGT AGS TGG 3’	

Table 4 (Continued)

Primer	Sequence	Description
rpoE A-up-*HindIII*	5’TTC AAGCTT TGT ATT ACG GCT ACC CGA CCA 3’	Amplification of 444-bp of upstream *rpoE*
rpoE B-Down-*AscI*	5’TTC GGC GCG CCG AA TTA TCG GTC AGT GCC CAT CAC 3’	
rpoE C-up-*AscI*	5’TTC GGC GCG CCG AA AAT GCT CGC ACG CCT GGG AGTT 3’	Amplification of 472-bp of downstream *rpoE*
rpoE D-Down-*HindIII*	5’ TTC AAGCTT TGC TGT GCA TGC AGC AGG CGA T 3’	
rpoN-F	5’ ATG ACT GAC ACC GAT ACC GTG 3’	Amplification of 820-bp of *rpoN*
rpoN -R	TTG CAT GGT CTC CGT AGS TGG 3’	
rpoN A-up-*HindIII*	5’TTC AAGCTT TGT ATT ACG GCT ACC CGA CCA 3’	Amplification of 458-bp of upstream *rpoN*
rpoN B-Down-*AscI*	5’TTC GGC GCG CCG AA TTA TCG GTC AGT GCC CAT CAC 3’	
rpoN C-up-*AscI*	5’TTC GGC GCG CCG AA AAT GCT CGC ACG CCT GGG AGTT 3’	Amplification of 426-bp of downstream *rpoN*
rpoN D-Down-*HindIII*	5’ TTC AAGCTT TGC TGT GCA TGC AGC AGG CGA T 3’	

3. Motility analysis

All of mutants and complemented strains were tested for their swimming motility compared with wildtype (strain 12-2). The assay was modified from Das *et al.* (2002). Swimming motility media was containing 2.4 g potato dextrose broth (Difco), 1 g KH_2PO_4, 0.057 g K_2HPO_4, 0.1 g yeast extract (Difco), 0.42 g $MgSO_4.7H_2O$, 3.0 g agar (Difco), 1 L distilled water, and adjust pH5.5. Swimming tube was inoculated with bacteria and incubated overnight culture on swimming motility media test tubes at 28^{o}C. The test tubes were then sealed with parafilm to prevent dehydration and incubated at 28^{o}C for 24 h. The swimming positive was observed by the bacterial cell moved from the strapped center. Bacterial strain lacking moved from the strapped center or just grown in the strapped center was checked three times to verify the swimming motility-minus phenotype.

4. Growth curve of bacterial strains

Bacterial strains were grown overnight in NGA and then were inoculated into LB with to a final OD_{600nm} of 0.1. Aliquots of 150 µL were used to fill the difference wells of 96-well polystyrene plate and incubated at 28^{o}C for difference periods (1, 3, 6, 12, 24, 48, and 72 h). To confirm similar bacteria growth, optical density at 600nm was measure before the adhesion assay was performed. To analyze bacterial growth in LB medium at 28^{o}C and 200 rpm were used. Aliquots of 150 µL were poured in different wells of a 96-well polystyrene plate and bacteria were incubated as previously described. The OD_{600nm} and attachment was measured as describe above.

5. Biofilm formation

Bacterial strains were grown overnight in NGA and then were inoculated into yeast minimal medium (YMM) broth to a final OD_{600nm} of 0.1. Aliquots of 150 µL were used to fill the difference wells of 96-well polystyrene plate and incubated at 28^{o}C for difference periods (1, 3, 6, 12, 24, 48, and 72 h).

The quantification of bioflim development and the adhesion of cells to an abiotic surface were determined, the medium were gently removed using a pipette, the 96-well plate was washed with 0.9% NaCl and stained using 0.1% crystal violet solution, incubated at 28^{o}C for 30 min and washed with distilled water. The crystal violet in each well was solubilized by adding 150 µL of 70% ethanol and quantified by absorbance at 570 nm (O'Toole and Kolter, 1998).

6. Protein analysis

This experiment was to determine the flagellin protein of *X. axonopodis* pv. *glycines* that were associated with pathogenesis of *X. axonopodis* pv. *glycines.*

6.1 Extraction of protein

X. axonopodis pv. *glycines* wildtype 12-2 and mutant strains, *flgC, fliC, fliD, fliC&fliD, fliD&fliC,* and *fliD&xagP* were grown in 500 mL LB at 28^{o}C for 48 h. Bacterial cells were harvested by centrifugation at 10,000 rpm at 4^{o}C for 10 min, and then filtrated through a 0.2 nm nitrocellulose membrane tube, precipitated the flagellin protein with 15% ammonium sulfate at 4^{o}C for 3 hour. Protein were harvested by centrifugation at 12,000 rpm at 4^{o}C for 30 min, following 15 mL of HEPES buffer was added to the protein precipitated, mixed well and then dialyzed protein filtrated through a 0.2 nm nitrocellulose membrane tube and incubated in HEPES buffer at 4^{o}C for 12 hour (Kladsuwan *et al.,* 2012).

6.2 SDS-PAGE proteins analysis

The flagellin protein was taken from 6.1. Flagellin proteins were separated by SDS-PAGE (Kladsuwan *et al.,* 2012). The gels were strained with Coomassie stain briefly described, remove SDS-PAGE gel from glass and rinse once in ddH$_2$O in a suitable container with a lid. Added enough Coomassie stain to cover the gel by ½ inch (~1.5 cm) and incubated at room temperature (30±3^{o}C) overnight. Destain solution to remove Coomassie stain from the container. Added fresh Destain solution to cover the gel by ¾ inch (~2 cm) and incubated the gel 3 times in the Destain solution for 3 hour on a rocking table.

7. Extracellular enzyme assays

In bacteria exoenzymes play an integral role in allowing the organisms to effectively interact with their environment. Many bacteria use digestive enzymes to break down nutrients in their surroundings. Once digested, these nutrients enter the bacterium, where they are used to power cellular pathways with help from endoenzymes.

Many exoenzymes are also used as virulence factors. Bacterial pathogens can use exoenzymes as a primary mechanism with which to cause disease, especially *X. axonopodis glycines*. The exoenzymes have an importance for *X. axonopodis glycines* to cause disease severity on soybean (Thowthampitak *et al.*, 2008).

7.1 Enzyme activity assays

To assess extracellular enzyme activity, all of mutant strains were grown in NGB at 28^{o}C overnight (to render either precise or broken cells), unless a different time is specified below. Cells were pelleted by centrifugation at 15,000 rpm for 10 minute, and the supernatants from precise-cell (to determine for enzyme secretion) and broken-cell cultures (enzyme production) were collected and sterilized by filtration (0.2 µm). Thirty µL of the filter-sterilized supernatant was placed in a 0.5 cm well, made using a cork borer, in an agar medium in a Petri plate. The medium composition, incubation conditions for assay plates, and detection conditions for each assay are specified below. Enzyme activity was estimated from the diameter of zones surrounding the culture supernatants of each strain. All experiments were done twice, once with four replicate plates and once with three replicate plates. The production of extracellular enzymes, including alpha-amylase, cellulases, endoglucanase, protease, pectate lyase, and polygalacturonase was investigated using the following methods and determined by using a spectrophotometry methods.

7.1.1 Cellulase activity

X. axonopodis pv. *glycines* mutant strains were grown in NGB at 28^{o}C for 24 h. Thirty µL of clarified supernatant was dropped into a 0.5 cm diameter hole in cellulase assay medium (0.1% carboxymethel cellulose, 25 mM sodium phosphate pH7, and 0.8% agarose) and incubated at room temperature. The cellulase plates were stained with 0.1% Congo red for 20 min and washed twice with 1M NaCl. Cellulase was visualized as white halos surrounding the wells.

For spectrophotometric determination of cellulase activity was performed according to Ghose (1987). Bacterial cultures in nutrient broth (NB) were centrifuged at 8000 rpm for 5 min to collect the supernatant with the secreted enzyme. Supernatant containing enzyme (0.5 mL) was added to 1 mL of 50 mM citric acid buffer (pH4.8) and incubated at 50°C for 60 min with filter paper added as substrate (Whatman No. 1 filter paper 1 cm x 6 cm). Three mL of dinitrosalicylic acid reagent (1% 3,5-dinitrosalycilic acid (Sigma), 1.2% NaOH, 0.05% sodium sulfate, and 20% potassium sodium tartrate tetrahydrorate) was added to the enzyme solution to stop the reaction, and the mixture was boiled for 5 min. The reaction mixture was transferred to an ice-cold water bath until measure. The cellulose activity was determined by measuring absorbance at 540 nm with spectrophotometer (UV-Visible Spectophotometer model Genesys^{TM} 10S UV Vis). The assay was done three times, with four replicate cultures in each assay.

7.1.2 Alpha-amylase activity

Alpha-amylase assay medium (0.5% yeast extract, 1.0% tryptone, 0.25% NaCl, 0.2% starch soluble and 0.8% agarose) was as described by Ray *et al.* (2010) with some modifications described by Lory *et al.* (1998). Thirty µL of clarified supernatant mutant strains was dropped into the 0.5 cm diameter hole in the agar medium and incubated at 28^{o}C for 24 h. Alpha-amylase were stained with potassium iodine for 10 min. Levels of alpha-amylase were assessed by measuring the diameter of clear zones.

For spectrophotometric determination of alpha-amylase activity was modified from Rick and Stegbauer (1974). Bacterial cultured in NB centrifuged at 8000 rpm for 5 min and supernatant including the secreted enzyme (0.5 mL) was transferred to 0.5 mL of 1.0% soluble starch solution in a test tube. The mixture was incubated at 60ºC for 10 min and then 1.0 mL of dinitrosalicylic acid was added to each tube. The test tubes were placed in boiling water for 5 min and cooled at room temperature. Then solution was diluted by adding 10 mL distilled water. The absorbance was determined at 540 nm using a spectrophotometer. The assay was done three times, with four replicate cultures in each assay.

7.1.3 Protease activity

Protease activity was determined by an agar plate assay. The test medium contained 1% skim milk, 1% tryptone (Difco), 0.5% yeast extract (Gibco BRL), 0.5% NaCl, and 1.5% agar. Thirty µL of clarified supernatant was dropped into a 0.5 cm diameter hole in the agar. Extracellular protease production was detected visually as clear halos surrounding the wells.

For spectrophotometric determination of protease activity was determined using the method of Satake *et al.* (1963). Briefly, after bacterial cultivation in NB medium was extracted with four volumes of 0.1% NaCl solution with shaking for 1 h at 25^{0}C. The reaction mixture included 0.1 g casein as substrate added to 10 ml 200 mM Mes buffer, pH 7.0. The solution was incubated at 60°C for 30 min, centrifuged at 10000 rpm for 15 min to collect the supernatant used as the protease substrate. A 20 µL trypsin solution was used as positive control by mixing with 180 µL of the casein solution mentioned above and incubated at 37°C for 15 min. Subsequently, 400 µL of 5% trichloroacetic acid (TCA) was added to end the enzymatic reaction. The reaction was mixed and centrifuged at 10000 rpm for 15 min. Protease activity was read at 280 nm with spectrophotometer (UV-visible spectophotometer model Genesys^{TM} 10S UV Vis) (Keay and Wildi, 1963). The assay was done three times, with four replicate cultures in each assay.

7.1.4 Endoglucanase

For measurement of endoglucanase activity all strains of *X. axonopodis* pv. *glycines* were grown in NGA supplemented with 0.125% carboxymethylcellulose (Barber *et al.*, 1997). After inoculation, plates were stained with 0.1% congo red for 30 min, rinsed with water, and washed twice with 1M NaCl. Endoglucanase activity was observed by radial diffusion of a pale yellow zone of clearing in contrast to a red background, as described by Gough *et al.* (1988).

For spectrophotometric determination of endoglucanase activity was modified from Bernfeld (1955). Bacterial culture incubated at room temperature for 24 h in NB was centrifuged at 8000 rpm for 5 min to collect the supernatant with the secreted enzyme; 0.5 mL was transfer to 0.5 mL carboxymethyl cellulose (CMC) (1% v/v) in 100 mm Borax-NaOH buffer, pH 11 and incubated at 50ºC for 30 min. The reaction was stopped by the addition of 2 mL of 3, 5-dinitrosalicylic acid reagent. The absorbance was determined at 546 nm using a spectrophotometer. The assay was done three times, with four replicate cultures in each assay.

7.1.5 Pectolytic activity

7.1.5.1 Pectolytic activity on potato

The putative gene *xagP* appeared to encode a pectate lyase, assays were done to measure pectolytic activity. Slices of potato tubers (*Solanum tuberosum*) were inoculated with cells of *X. axonopodis* pv. *glycines* wildtype 12-2, mutant 12-2 *fliC*::Km^r, 12-2 *xagP*::Gen^r, and double mutant including 12-2 *fliC&fliD*, 12-2 *fliD&fliC*, and 12-2 *fliC&xagP* that were grown 24 h on NGA. A toothpick was dipped in the bacterial culture and then stabbed into the surface of the potato slice. The slices were incubated on moist filter paper at 28°C in Petri dishes and the development of soft rot was evaluated after 24 to 48 h.

7.1.5.2 Pectate lyase activity assay

The mutants were grown in minimal medium (MM) consisted of $Na_2HPO_4.7H_2O$ 64g, KH_2PO_4 15g, NH_4Cl 2.5g, and adjust to 1L with ddH2O (Daniels *et al*., 1984) supplemented with 1% polygalacturonic acid (PGA) and subjected to the spectrophotometric assay. The cell density of an overnight culture grown in MM was measured at an optical density of 600 nm (OD_{600}nm), and the supernatant was obtained by centrifugation at 15,000 rpm for 2 min. For bacteria cultured in MM supplemented with PGA, cells were separated from the supernatant by centrifugation at 6,500 rpm for 10 min and suspended in 0.5 mM potassium phosphate buffer to measure cell density at OD_{600}nm. Pectate lyase activity was measured at OD_{230nm} by using the culture supernatants (Nasuno and Starr, 1967). One unit of activity causes the liberation of 0.5 µmole of unsaturated product per minute. The assay was done three times, with four replicate cultures in each assay.

7.2 Enzyme production assay

To assess extracellular enzyme production, all of mutant strains were grown in NGB at 28^oC overnight, unless a different time is specified that mentioned above. Cells were pelleted by centrifugation, and sonication. The production of extracellular enzymes, including alpha-amylase, cellulases, endoglucanase, protease, pectate lyase, and polygalacturonase was investigated using the following methods above number 7.1.

7.3 Analysis of enzymatic gene expression

To further study if *fliC* and *xagP* regulated up or downstream of certain enzyme synthesis including alpha-amylase, cellulase, and protease; analysis of related genes transcription in mutant and wildtype strains was performed by RT-PCR. The RNA extraction was used RNeasy Mini Kit protocol (QIAGEN®) (Nuyts *et al.*, 2001). cDNA concentrations were normalized base on primers for the constitutively expressed gene encoding ribosomal protein. The RT-PCR primers included forward -

alpha-amylase -5’ ACA AGC CGT TCC GCT CGG 3’ and reverse - alpha-amylase - 5’ AAG TCC ACG CCG AAC TCG GT 3’; forward-cellulase-5’ ATC CAC GTG CAG TTC GAC CTT 3’reverse-cellulase-5’ ACA GCG ATT GAT GAA GAC CGC A 3’; forward- protease -TGG CTT CGG CCA GGT ACA G 3’ and reverse- protease -5’ CGA AGC CAC GCT GA 3’(reference sequence, accession number AE008923.1), primed the amplification of a 312; 408; and 315 bp fragments of the genes respectively. A 16S rRNA real-time PCR, designed to detect genomic DNA from equine commensal bacteria, was run in parallel with the real-time PCR to ensure successful DNA extraction (Wakeley et al., 2006). A total of 30 amplification cycles were performed in an automated thermocycler (P-100). Each cycle consisted of 20 sec of denaturation at 95°C, 30 sec of annealing at 55°C, 6 min of extension at 65°C, and the last extension step at 68°C was extended to 20 min. Amplified DNA was determined by agarose gel electrophoresis on a horizontal, 0.8% agarose gels in Tris/Borate/EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) at 70 volts constant for 4 h 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). Change values for gene expression from RT-PCR analysis were measured. Extraction of DNA from agarose gel used MinElute Gel Extraction Kit (QIAGEN$^{®}$) and total DNA quantity evaluated by spectrophotometry using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) were then carried out.

8. Pathogenicity test and bacterial growth on soybean

Soybean susceptible cultivar to *X. axonopodis* pv. *glycines* pathogen cv. Spencer was used in this experiment. The soybean seed surface sterilized by treatment with 95% ethanol (v/v) for 20s, followed by soaking in 20% bleach (v/v) for 20 min. Seeds were washed with sterile distilled water 5 times in order to remove excess bleach. The seeds were then air dried by placing them in the laminar flow hood for 5 min. To produce soybean, 2 viable seed were planted in 30 cm-diameter pots containing a steam-pasteurized potting medium (Sharpsburg silt clay loam, vermiculite, and sand mixed in equal volumes), and the seedlings were grown in a greenhouse for 2 weeks. The pots were watered daily with a nutrient solution (20-10-20). In all experiments, pots of soybean plants were kept in a greenhouse with a 12-h photoperiod (25^{o}C and 60-75% relative humidity during the light period, 15^{o}C and >93% relative humidity during the dark period).

Virulence of wildtype, single mutants (*flgC, fliC, fliD, xagP*) and double mutant (*fliC&fliD, fliD&fliC, fliD&xagP*), and complemented strains were assessed on soybean cultivars Spencer by infiltration and spray inoculation technique. Disease severity was assessed using a scoring method (Prathuangwong *et al.*, 1993). Bacterial growth was also assessed within the infiltrated zone. Leaf discs, 5 mm in diameter, were cut from the center of the infiltrated zone at daily up to 7 days post-infiltration. Samples were placed individually in 1 mL sterile water, triturated, and dilutions plated on NGA. Ten leaves of ten plants were evaluated for each strain. Moreover, diameter of infiltrated zone and the number of pustules surround the infiltrated zone was observed (Athinuwat *et al.*, 2009). The experiment was repeated three times. The results were statistically analyzed using SAS 9.1.2 (SAS Institute Inc., Cary, NC, USA).

9. Hypersensitive response induction

The ability of wildtype, single mutants (*flgC, fliC, fliD)* and *xagP*, double mutant (*fliC&fliD, fliD&fliC, fliD&xagP*) and complemented strains to induce an HR was assayed on soybean cultivars Spencer and non-host three species of tobacco (*Nicotiana glauca*, *N. rustica*, and *N. tabacum* cv. Xanthi), tobacco, and chilli as previously described (Kaewnum *et al.*, 2006). Bacterial suspensions at cell densities of about 1×10^{8} CFU/mL (for soybean plant) and $5.0\text{x}10^{9}$ CFU/mL (for tobacco plant) were infiltrated into the leaf mesophyll. Test plant leaves were inoculated on the leaves with a pin and then pressing the leaves with a tuberculin syringe against the hole in the top of the leaf while closing the hole and supporting the bottom of the leaf with a finger. Infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h after inoculation. The experiment was repeated three times.

10. The role of sigma factor regulation

To complement the relatedness between *xagP* and flagellin genes that affected extracellular enzyme activity, the alternate sigma factor genes *rpoE* and *rpoN* predicted to Xag linking genes were investigated. Most of sigma factors are involved in the environmental stress responses (Mittenhuber, 2002) however, they may be directly essential for virulence that depend on host plants (Kovacikova and Skorupski, 2002). The sigma E and N are known to regulate various functions including transcriptional regulation involved in bacterial pathogenicity (Kazmierczak *et al.*, 2005). Moreover, sigma E; and sigma N have been reported to regulate directly the pectate lyase and other extracellular enzyme activities such as protease (Chatterjee *et al.*, 2002); and the flagella biogenesis and motility respectively.

10.1 The *rpoE* and *rpoN* mutants of strain 12-2

The GenBank accession number for the sequence of *rpoE* and *rpoN* is *X. axonopodis* pv. *citri* the accession no. NC_003919. A Tn5 insertion was introduced via gene deletion as earlier described (Ichinose *et al.*, 2003b). Description of two genes was confirmed by PCR with primers rpoE-F and rpoE-R and rpoN-F and rpoN-R for *xagrpoE* and *xagrpoN* respectively (Table 4), the expected Tn5 insertions in *rpoE* and *rpoN* were selected for function study.

10.2 Characterization of the sigma factor mutants

The *rpoE* and *rpoN* mutants were tested for their pathogenicity on host (full virulence) and nonhost (HR). Influence of these sigma factors on regulation of *xagP* and flagellin genes that related with extracellular enzyme activity were evaluated as above description in materials and methods numbers 3 to 7. For the swimming assay was modified from number 3. Briefly, swimming motility media (with 0.4% agar) plate was inoculated by dropped with a 3 μL of 0.05 OD_{600} bacterial suspensions from overnight culture on swimming motility media plates at 28^{o}C. The plates were then sealed with parafilm to prevent dehydration and incubated at 28^{o}C for 24 h. The swimming positive was observed by the bacterial cell moved from the dropped center. Bacterial strain lacking moment from the dropped center or just grown in the strapped center was checked three times to verify the swimming motility-minus phenotype.

10.3 Analysis of gene expression

To further evaluate and confirm the linking of different pathogenicity factor, gene expression in *xagrpoE* mutant was determined by RT-PCR. The RNA extraction using RNeasy Mini Kit protocol (QIAGEN$^{®}$) was conducted as mentioned above. The real-time PCR targets the *xagP; flgC; fliC;* and *fliD* genes expression in *xagrpoE* mutant. Primers forward -xagP-5’AGA CGT TCG GCG ATC TGT TTG T3’ and reverse -xagP-5’ACC TGG TCC AGG AAA CCT ATC T3’; forward-flgC-5’TCC AAT CTG GCC AAT GCC GAT T3’reverse-flgC-5’CCT TGG CGG TAT TGA GCA TTT C3’; forward-fliC-5’AGT CAA GCA GCT GAC CTC TGA A3’ and reverse-fliD-5’TCT GGT CCA GCT TGT TGA3’; and forward-fliD-5’ATC AGA GCG TCA CCG TAG ACA T3’ and reverse -fliD-5’CCA GAT TGA ACT TGG TGC CTT C3’ (reference sequence, accession number NC_003919), that prime the amplification of a 331; 397; 380; and 379 bp fragment of the gene *xagP, flgC, fliC,* and *fliD* genes, respectively. The conditions of RT-PCR and amplified DNA were determined by agarose gel electrophoresis that mentioned in number 7.3.

RESULTS AND DISCUSSION

Results

1. Strain of *X. axonopodis* pv. *glycines* 12-2 wildtype and host plant inoculation

The *X. axonopodis* pv. *glycines* strain 12-2 originated from soybean production areas of Nakhonrachasrima, Thailand was revived from stock cultures of the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University and shown the colonies on nutrient glucose agar are yellow and smooth rimmed (Figure 3A); and shown to cause pustule disease on susceptible soybean cultivar Spencer. Symptoms were small, yellow-green spots with reddish-brown centers on the upper leaf surface at 3-5 days after inoculation. The central portion of each spot appeared slightly raised and developed into a small pustule, especially on the underside of the leaves. Several infections on the same leaf produced a large, yellow to brown area with small, and dark brown spots. The brown, necrotic areas on older leaves might break up and caused a ragged appearance (Figure 3B). Symptoms begin as small, light green spots that do not appear water-soaked. Light-colored raised blisters (pustules) often develop in the center of lesions on both the upper (Figure 3B) and lower surface of leaves (Figure 3C). The lesions can grow together into large, irregular patches of dead tissue. Loss of tissue in the infected areas may give the leaves a ragged appearance. Small raised spots may also develop on pods. Early symptoms of bacterial pustule look similar to bacterial blight. However, bacterial pustule can be differentiated in that the lesions do not appear water-soaked, there is less chlorosis around lesions, and by the formation of tiny tan bumps in the center of the lesions. The pustules can be mistaken for those of soybean rust, however, bacterial pustules will not have a natural opening in the pustules or masses of spores like those of soybean rust (Figure 3D). These features can only be seen under magnification (20 X or higher) (Hartman and Wang, 1997).

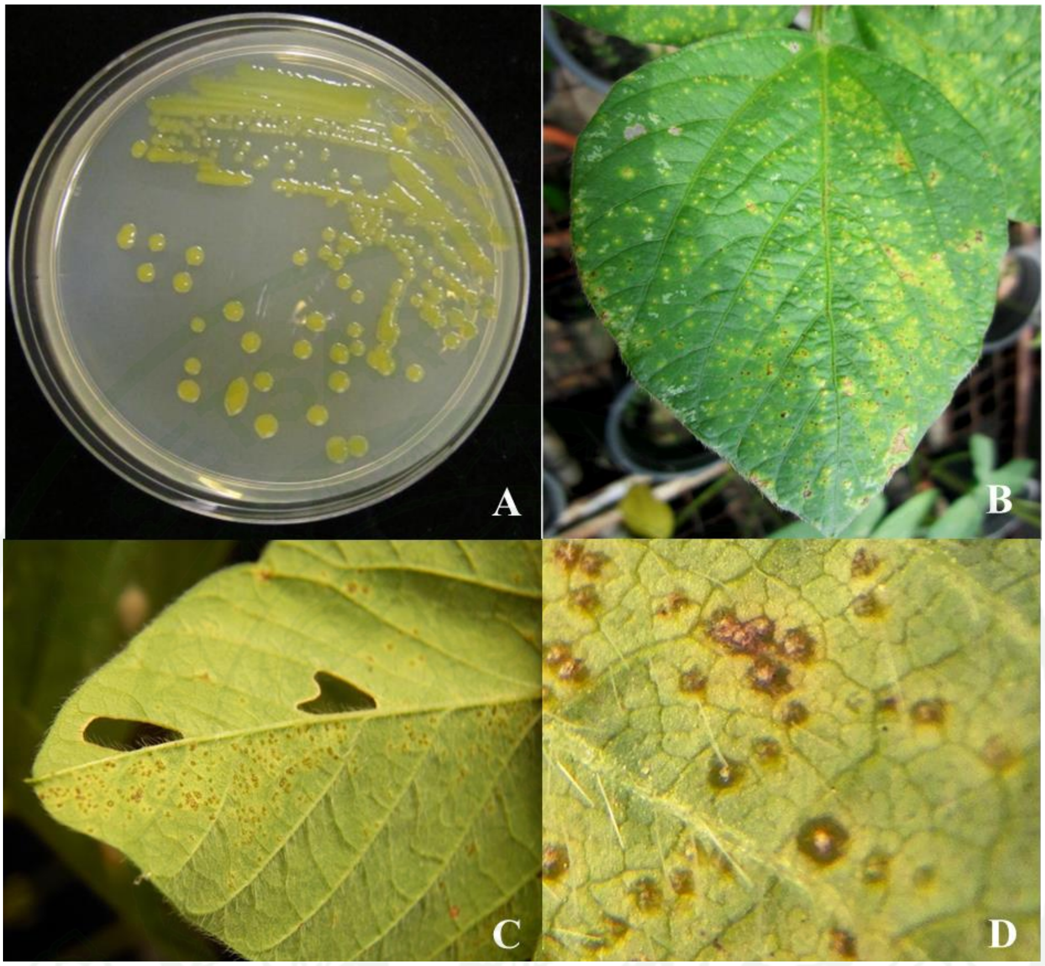

Figure 3 Colonies of *Xanthomonas axonopodis* pv. *glycines* 12-2 on nutrient glucose agar are yellow and smooth rimmed (A) caused bacterial pustule on soybean leaves, typical symptoms of bacterial pustule are small yellow–green spots with brown centers, noticeable mainly on the upper surface of new leaves (B-D). Bacterial pustule upper leaf surface (B), under leaf surface (C), and close-up of the raised pustules in the center of lesions (D).

2. Analysis of gene abolished

X. axonopodis pv. *glycines* mutants, 12-2 *flgC*::Km^r, 12-2 *fliD*::Km^r; and 12-2 *fliC*::Gen^r, 12-2 *xagP*::Gen^r were selected to amplify the fragment of kanamycin; gentamicin gene by the primer flgC-A and flgC-D, fliD-A and fliC-D; fliC-A and fliC-D, xagP-A and xagP-D, respectively compared to wildtype strain. The 1200-bp of kanamycin gene was detected from 12-2 *flgC*::Km^r, and 12-2 *fliD*::Km^r mutants and the 1200-bp of Gen^r were detected from 12-2 *fliC*::Gen^r and 12-2 *xagP*::Gen^r mutants. In contrast, the wild type could not amplify DNA product of PCR analysis (Figure 4). Also, the *flgC, fliC, fliD,* and *xagP* in wildtype showed size 408; 1,497; 1,559; 1,400-bp which detected by PCR with flgC-A and flgC-D; fliC-A and fliC-D; fliD-A and fliC-D; xagP-A and xagP-D, respectively (Figure 4).

3. Motility analysis

To clarify the role of flagella of *X. axonopodis* pv. *glycines* strain 12-2, we generated 6 flagella-defective mutants including *flgC, fliC, fliD, fliC*&*fliD, fliD*&*fliC,* and *fliD*&*xagP* mutants, by homologous recombination. The 4-flagella mutants; *flgC, fliC, fliC*&*fliD,* and *fliD*&*fliC* were completely lost swimming motility and *fliD* and *fliD&xagP* motility were reduced. Because *fliC* encodes the flagellin protein itself, the *fliC* mutant did not produce flagellin. On the other hand, *fliD* encodes the hook associated protein2 (HAP2)-capping protein that functions to polymerize monomer flagellin to form the filament at the top of the flagellum. Thus, the *fliD* mutant did not form a flagellum but secreted a large amount of monomer flagellin into the culture medium (Shimizu *et al.,* 2003). When we inoculated soybean leaves with *flgC, fliC, fliC*&*fliD,* and *fliD*&*fliC* mutants, the mutants failed to spread disease symptoms. Moreover, the deletion of each single flagella gene (*flgC* or *fliC* or *fliD*) resulted in reduced virulence on soybean plant. The responses of soybean leaves to inoculation with the flagella-defective mutants were distinctly different. The mutant strains caused limited and delayed symptoms, whereas the *fliD* mutant caused reduced symptoms when compared with wildtype.

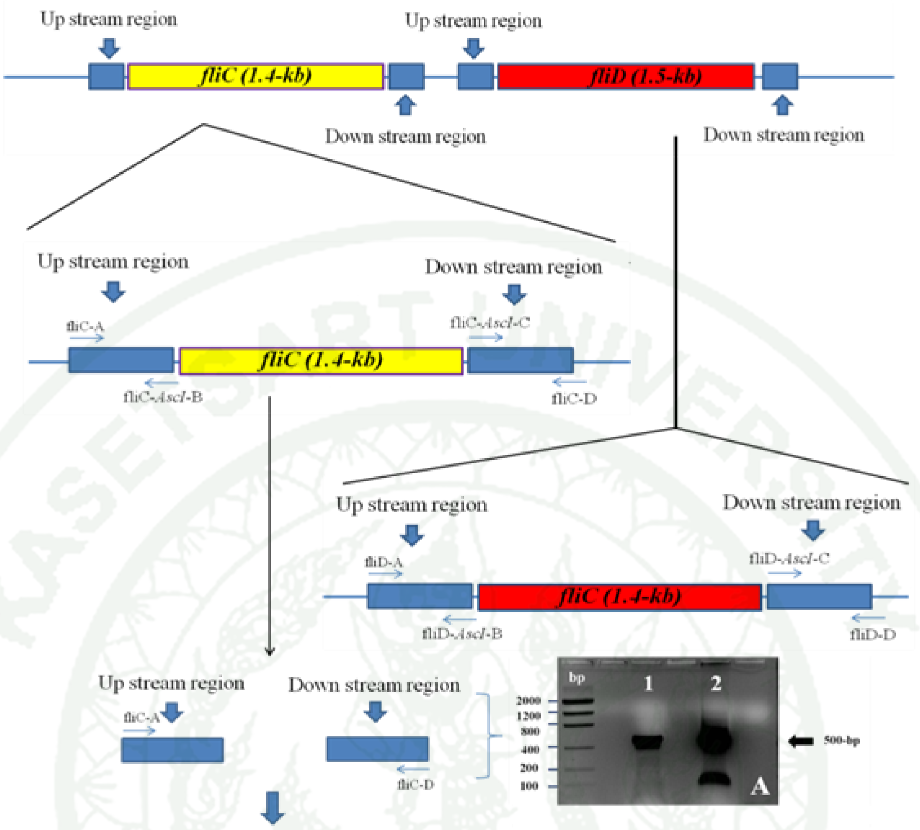

Figure 4 The diagram for deleted mutation, up and downstream regions of *fliC* (A); lane 1= up stream region and 2=downstream region genes 500-bp product was detected in *Xanthomonas axonopodis* pv. *glycines* 12-2. B= up and downstream regions of *fliC* was merge with fliC-A and fliC-D primers, amplified 1 kb polymerase chain reaction (PCR) product. The 1 kb sequence of up and downstream regions of *fliC* in pUC19 vector was digested with *Asc*I. C= digestion of pUC19-FLIC was inserted kanamycin gene shown fragment size 3.6 kb, lane 1-4=pUC19-FLIC with kanamycin inserted. D= PCR amplification of *fliC* gene from *fliC* mutant (1.2 bp kanamycin gene inserted) of *X. axonopodis* pv. *glycines* 12-2 with primer *fliC*-F and *fliC*-R (lane 1 to 3) compared with 1.4-kb fliC gene from wildtype (lane 4 and 5). The PCR products were separated by electrophoresis on a 0.8% agarose gel.

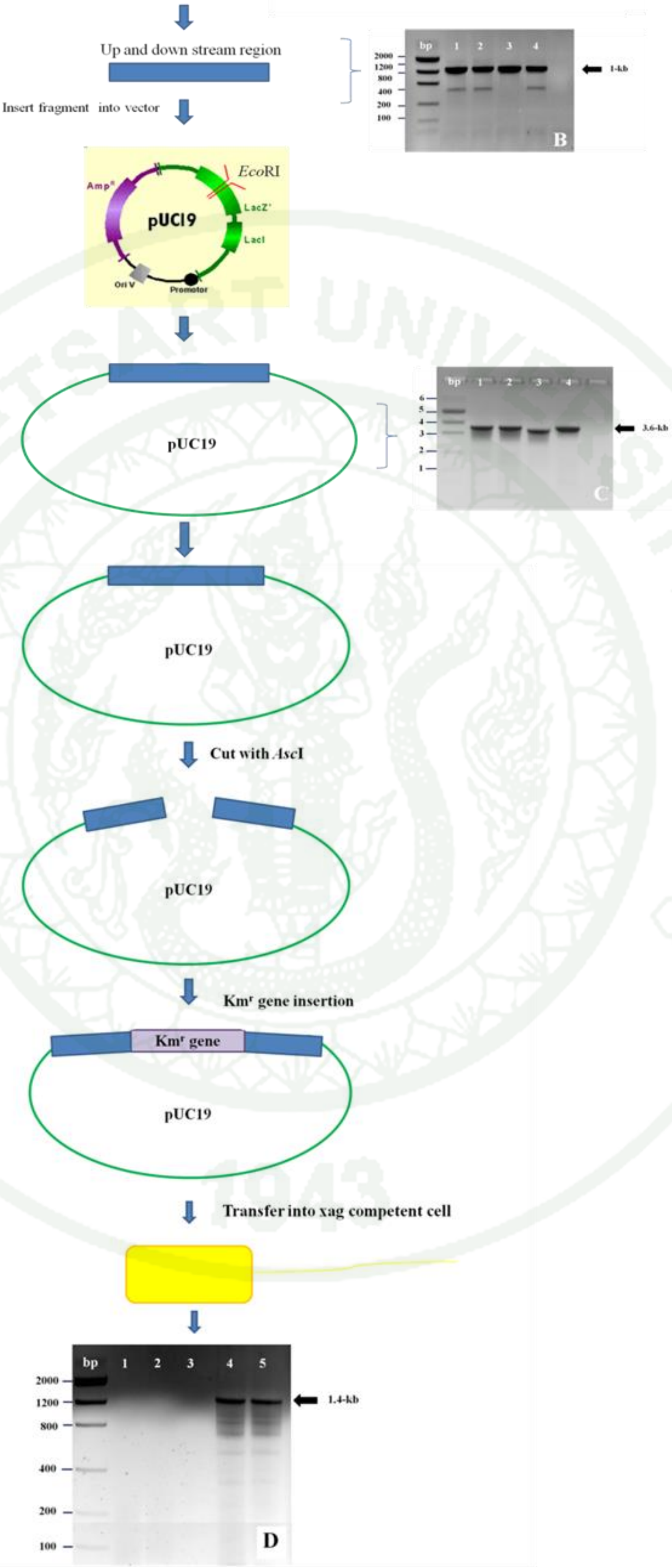

Figure 4 (Continued)

As shown in this study, flagella seem to be important for the virulence of *X. axonopodis* pv. *glycines* strain 12-2 on soybean plants. Based on the results obtained in this study, we discuss the correlations between motility, flagellin production, and virulence of bacteria on host soybean plants. The flagellum is the major component for swimming motility and the *flgC, fliC,* and *fliD* play the importance role of swimming and development of pustule disease on soybean (Figure 5).

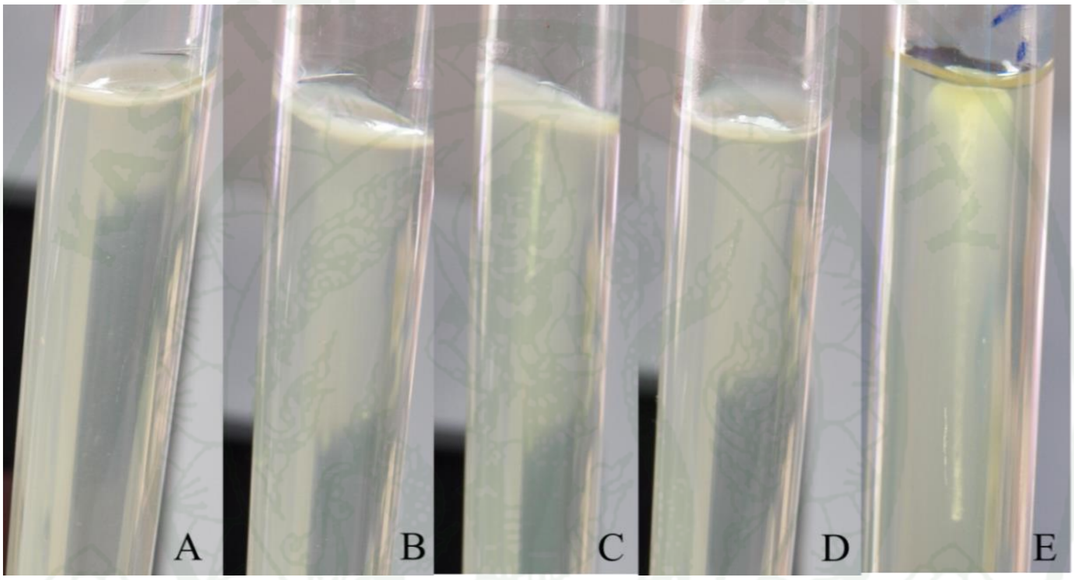

Figure 5 Motility of mutants; *flgC* (A), *fliC* (B), *fliD* (C) and *fliC&fliD* (D) compared to wildtype 12-2 (E) after incubated at 28°C for 24 h.

4. Extracellular enzyme assay

4.1 Enzyme activity assays

Due to the influence of flagella genes *(flgC, fliC,* and *fliD),* and *xagP* genes on exoenyzme productions and the important role of pectate lyase (Kaewnum *et al*., 2006) in the virulence of *Xanthomonas* spp., wildtype and mutant strains including single gene deleted mutants; *flgC, fliC, fliD, xagP* and double gene deleted mutants; *fliC&fliD, fliD&fliC,* and *fliD&xagP* were determined for the exoenzyme productions. The mutant strains *xagP* and *fliD&xagP* completely lost ability to produce pectinase and pectate lyases (Figure 6, 7 and Table 5) whereas *flgC, fliC, fliD, fliC&fliC,* and *fliD&fliC* expressed only pectinase and pectate lyases reduction. Our results indicated that XagP proteins might coordinate expression with *fliC* or translocation of pectate enzymes via flagella machinery. *FlgC, fliC, fliD,* and *xagP* mutants also altered the production of extracellular enzymes including cellulase, alpha-amylase, endoglucanase, and protease (Table 5 and Figure 6, 7, and 8).

Table 5 Activity of exoenzymes of *Xanthomonas axonopodis* pv. *glycines* wildtype and mutants after incubated at 28^{o}C for 24h

Strain	**Diameter of clear zone (cm)**[1,2]				
	Al	**Ce**	**En**	**Pe**	**Pr**
12-2	2.3a	2.0a	2.7a	1.4a	1.9a
flgC mutant	1.9a	1.6a	2.3a	0.9b	1.5a
fliC mutant	1.9a	1.5a	2.5a	0.8b	1.6a
fliD mutant	2.1a	1.8a	2.5a	1.0ab	1.6a
xagP mutant	2.3a	1.9a	2.3a	0.0c	1.7a
fliC&fliD mutant	1.2b	0.8b	0.0c	0.6b	0.8b
fliD&fliC mutant	1.1b	0.8b	0.1c	0.7b	0.8b
fliD&xagP mutant	2.0a	1.8a	2.0b	0.0c	1.7a
CV	26.9	33.8	67.5	79.2	31.2

[1] Overnight cultures in NGB were filtrated and placed in 0.5-cm diameter wells in agar media to detection of each exoenzyme. Enzyme activity was estimated from the diameter of zones surrounding each well. A value of 0.5 cm, the diameter of the well, indicates that no enzyme activity was detected. All experiments were done twice, once with four replicate plates and once with three replicate plates.

[2] Al= Alpha-amylase, Ce= Cellulase, En= Endoglucanase, Pe= Pectate lyases, and Pr= Protease

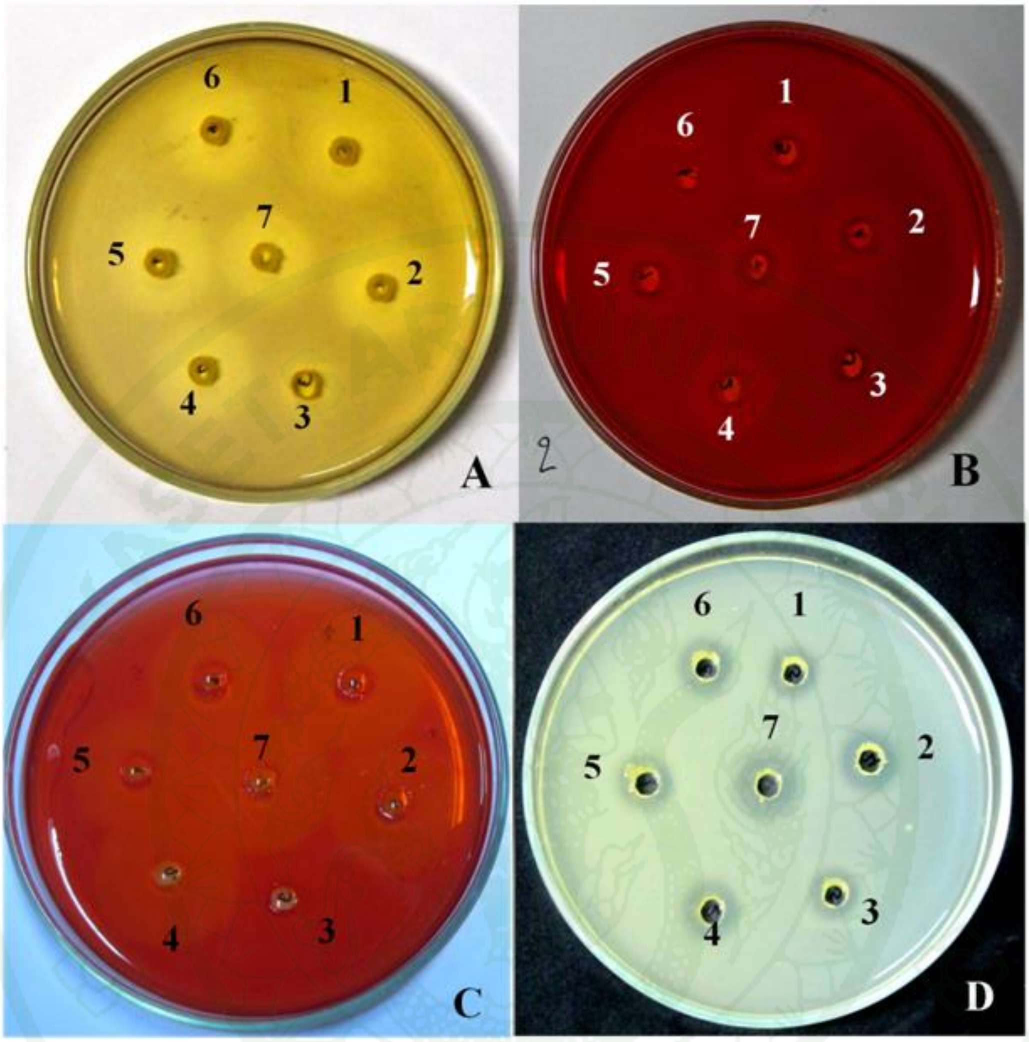

Figure 6 Exoenzyme activity of *Xanthomonas axonopodis* pv. *glycines* 12-2 mutants; 1=*fliC*, 2=*fliD*, 3=*xagP*, 4= *fliC*&*fliD*, 5= *fliD*& *fliC*, and 6= *fliD*&*xagP* compared to 7=wildtype 12-2. Filtrated cell were placed in wells in assay media to assess cellulase (A), alpha-amylase (B), endoglucanase (C), protease (D) activities. Assays were done twice, once with four replicates and once with three replicates.

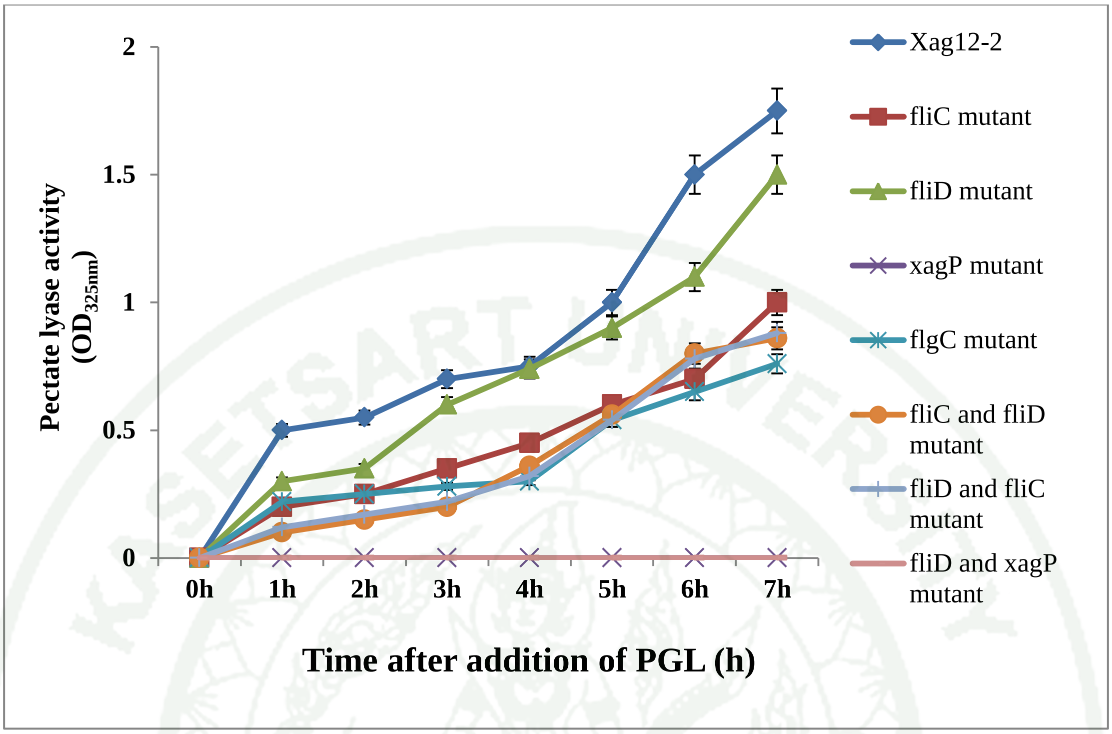

Figure 7 Pectate lyase activity of *Xanthomonas axonopodis* pv. *glycines* wildtype and mutants was measured by spectrophotometrically according to Materials and Methods described above. One unit of pectate lyase activity is defined by the amount of catalyzation of the cleavage of polygalacturonic acid with an increase in absorbance at a wavelength of 230 nm that is equivalent to 0.2 within 10 min under standard conditions.

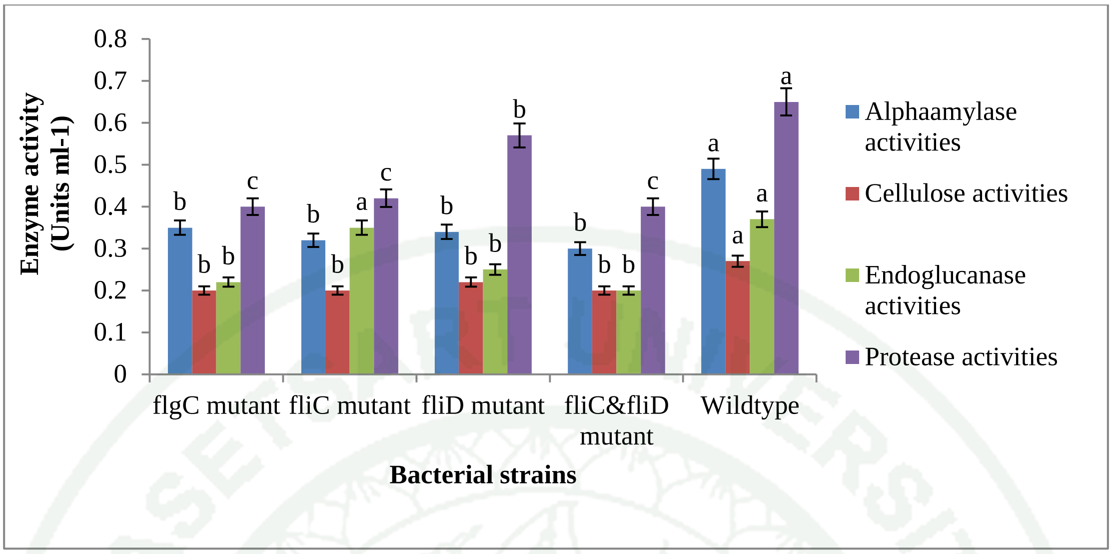

Figure 8 Flagella mutants exhibited reduced alpha-amylase, cellulose, endoglucanase, and protease activities compared to the wildtype strain 12-2. Each datum point represents the mean of results from three individual experiments, each with ten replicate samples. Bars represent standard deviation of the means.

4.2 Enzyme production assay

Supernatants of sonicated cells from wildtype and 7 mutant cultures were assayed for altered levels of 5-extracellular enzyme production using a spectrophotometrically assay. All of mutant especially double mutant strains showed decrease in all enzyme production. Of the mutant strains, *xagP* and double mutant *fliD&xagP* were found to be poor producer of the pectate lyase; whereas 5 mutants were significantly decreased in alpha-amylase production compared with the wildtype (Figure 9). Significant differences in cellulase, endoglucanase, and protease levels were also observed among 7-mutant strains. The decrease in these extracellular enzyme productions could be confirmed and explained by a reduction in transcription of involved genes in the target mutants. The RT-PCR to determine whether mutation of *xagP* and *fliC* caused a reduction in mRNA accumulation of any enzyme genes was further investigated.

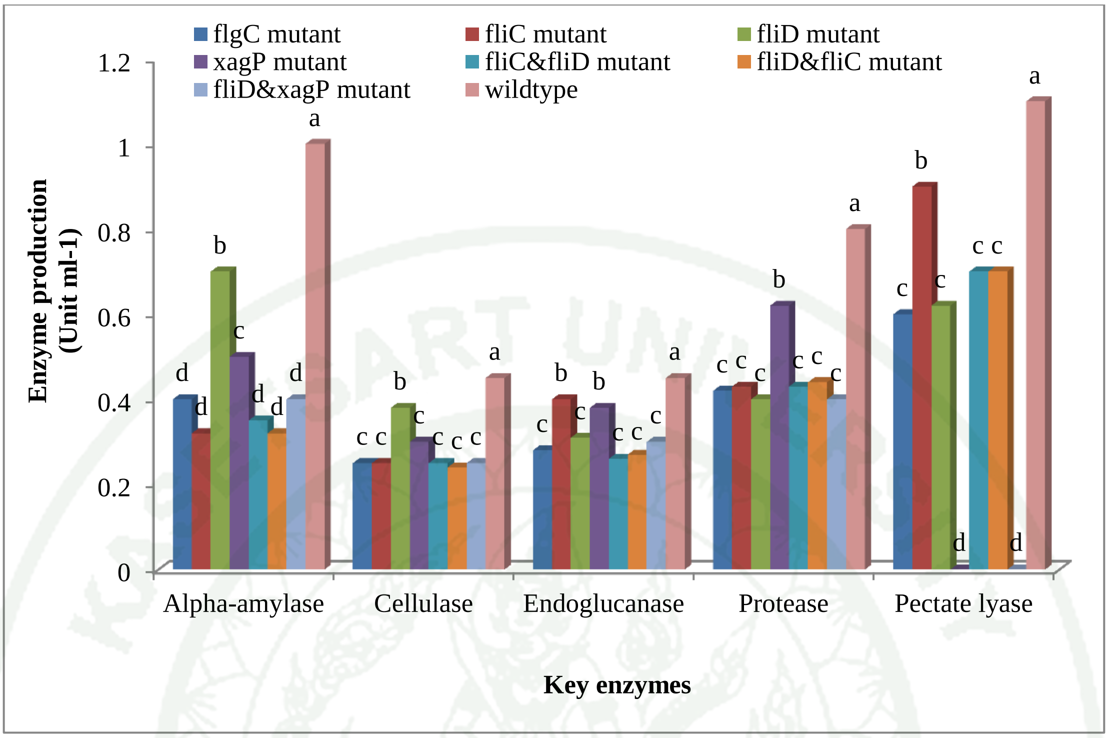

Figure 9 Enzyme production of *Xanthomonas axonopodis* pv. *glycines xagP* and flagella gene mutants. Enzyme production was measured spectrophotometrically according to methods described in Materials and Methods.

4.3 Analysis of enzymatic gene expression

In this study, the above plate activity and spectrophotometric assays, *xagP* and *fliC* mutant cultures were reduced in alpha-amylase, cellulase, and protease. To determine whether mutation of *xagP* and *fliC* caused a reduction in mRNA accumulation of these enzyme genes, RT-PCR and NanoDrop analyses were conducted for gene expression. The result showed that the reduction in mRNA levels for alpha-amylase, cellulase, and protease genes were observed. The *xagP*; and *fliC* mutant strains significantly decreased in mRNA levels of alpha-amylase, cellulase, and protease genes with 11.6, 16.6, and 19.9; and 3.6, 25.8, and 10.1% respectively, compared to wildtype (Figure 10). The *xagP* and *fliC* mutants had significantly lower these enzyme activities, they likely regulated one or more of the involved genes encoded by *X. axonopodis* pv. *glycines* 12-2 with direct (transcriptional activation) or indirect (another regulator suppression) regulation. Extensive works need to be elucidated in the future for mechanism that XagP and FliC affected these extracellular activities.

The data obtained confirmed the *xagP* not only affected pectate lyase production but also other key enzymes involved in virulence and pathogenicity factors of Xag 12-2 including; alpha-amylase, cellulase, and protease. The production of multiple enzymes that differ in this characteristics will enhance the bacterial ability to adapt to environmental modification (Ralston, 2008). Phytopathogenic bacteria generally produce a pool of enzymes for success infection of the plant tissues. Loss of or reduced enzyme activity therefore, they fail to cause disease on their host plants (Agrios, 2005). In this study, a reduction in alpha-amylase and protease activities from *xagP* and *fliC* mutants was similar but significant reduction in these two-enzyme-mRNA accumulations in *xagP* mutant was found (Figure 10). Generally, the reduction in enzyme activity is relative to reduction in mRNA levels of various genes involved (Zaidi *et al.*, 2004), *fliC* linkly affected expression of a few gene clusters involved. *FliC* thus, affected these enzyme secretions than production in this experiment. However, *Jahn et al.* (2008) reported that if relative expression levels of mRNA and the corresponding enzyme activities were examined, unpredictable results sometime have been obtained (Anderson and Seilhamer, 1997; Glanemann *et al.*, 2003; Gygi *et al.*, 1999).

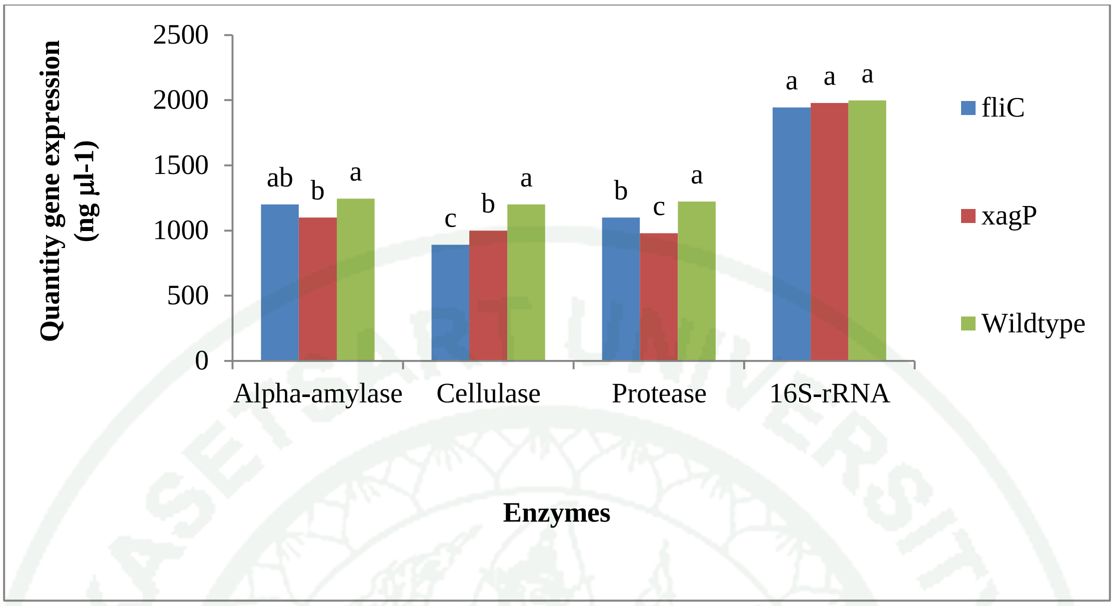

Figure 10 Expressions of alpha-amylase, cellulase, protease genes and 16S-rRNA genes, as internal standards in *xagP* and *fliC* mutants compared to wildtype was investigated by RT-PCR and NanoDrop analysis that mention in Materials and Methods.

5. Biofilm and adhesion quantification

The initial disease induction may depend on bacterial population that attached and development of biofilm on leaf surface. To examine the roles of flagella in the involvement of biofilm information in bacterial pustule disease, the ability of *X. axonopodis* pv. *glycines* to develop a biofilm has been determined on a 96-well polystyrene plate. We further characterized the development of biofilms in newly generated flagella mutants on motility media. When *X. axonopodis* pv. *glycines* 12-2 (wildtype) was cultured in yeast minimal medium (YMM), they formed visible biofilms on wells of a 96-well polystyrene plate with continuous agitation. The *flgC, fliC, fliD, xagP, fliC&fliD, fliD& fliC,* and *fliD&xagP* mutants were exhibited significantly reduced biofilms on similar surfaces and confirmed for bioflim formation on glass slid under compound microscope (Figure 11, 12). These results suggest that flagella play an important role in biofilm formation by causing more cells forming larger biofilm. These results indicated the mechanism of *flgC, fliC, fliD,* and *xagP* genes in *X. axonopodis* pv. *glycines* that are implicated in movement promotion, colonization, and virulence factor translocation in development of pustule disease. Moreover, no information is available about the role of motility in biofilm formation by *X. axonopodis* pv. *glycines* 12-2 and in turn the contribution of the biofilm to pathogenicity. To elucidate the role of flagella or motility in biofilm formation in *X. axonopodis* pv. *glycines* 12-2, we tested deletion mutants of all mutants and complementary mutants, for their ability to form biofilms in microtiter plates. The *flgC, fliC, fliC&fliD,* and *fliD&fliC* mutants were found to be nonmotile, whereas the *fliD* and *fliD&xagP* mutants were reduced motility. The results indicate that motility but not the physical presence of flagella is necessary for the formation of biofilm by *X. axonopodis* pv. *glycines* 12-2. We also raised the possibility that flagella-mediated motility may not be directly involved in pathogenicity but may be involved in the formation of a biofilm, a factor required for pathogenicity (Hossain *et al.,* 2005; Hossain and Tsuyunu, 2006; O'Toole *et al.,* 1999; O'Toole *et al.,* 2000). Recent molecular and genetic studies have identified several genes whose products are thought to be important for biofilm formation. Flagella or twitching-mediated motility has been reported to be important for free swimming cells of several bacterial species to initiate the formation of biofilms (O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Watnick *et al.,* 1999). In addition, the production of EPS (Davies *et al.,* 1993; Boyd and Chakrabarty, 1995; Davies and Geesey, 1995; Yildiz and Schoolnik, 1999) and outer membrane proteins (Danese *et al.,* 2000) was also shown to be required for biofilm formation. The catabolite repressor control (Crc) protein, which acts as a positive factor to induce the expression of many genes including those for carbon metabolism, was also reported to be necessary for biofilm formation in *Pseudomonas aeruginosa* (O'Toole *et al.,* 2000).

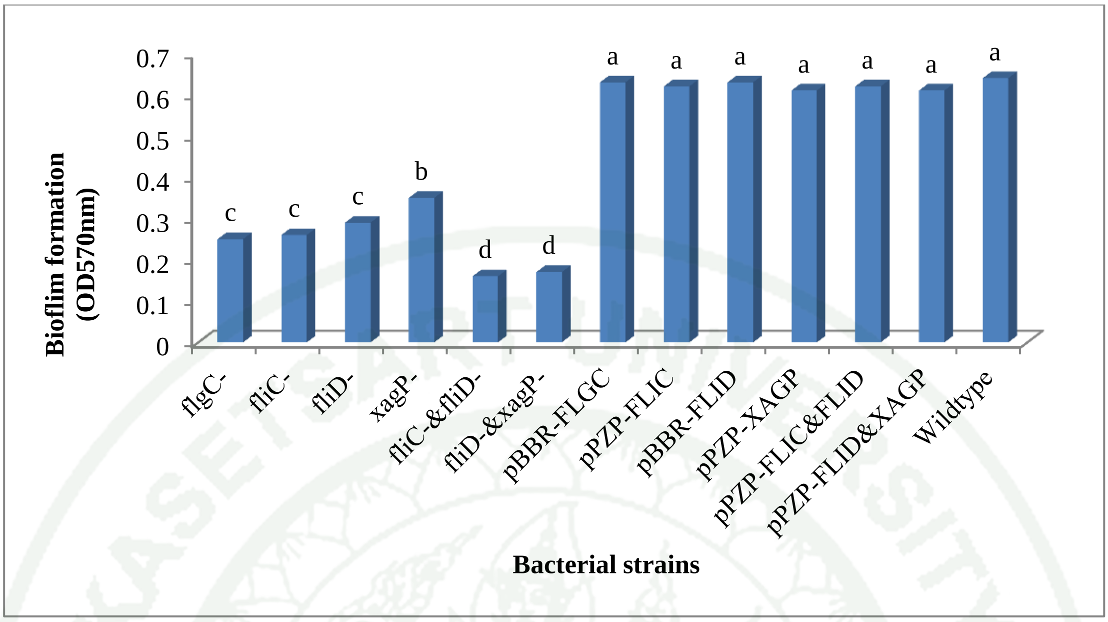

Figure 11 Comparison of the total OD_{570nm} of suspension of wildtype, mutants, and complemented mutants on bioflim formation in yeast minimal medium (YMM medium) at 28°C for 4 days.

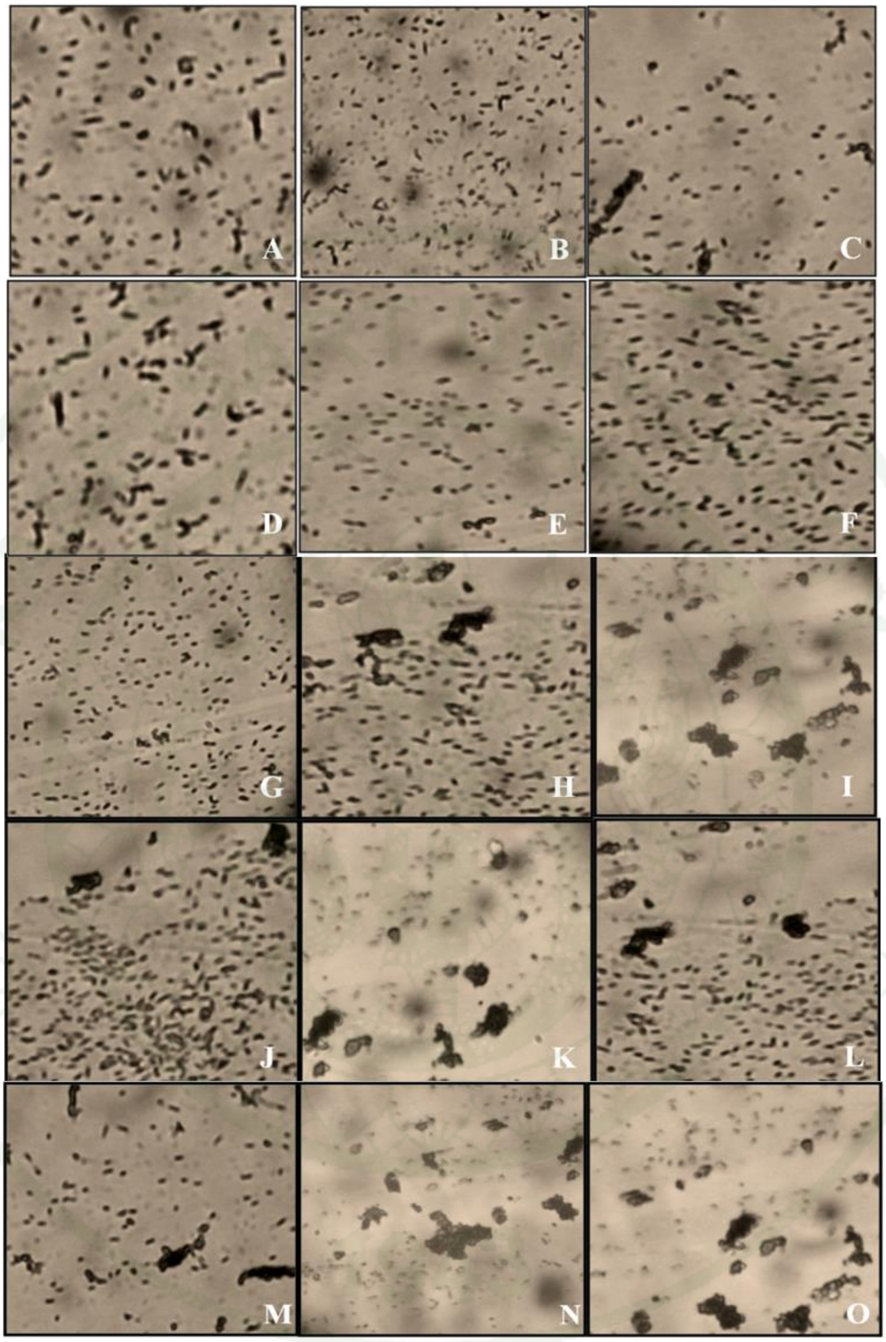

Figure 12 Comparison of bioflim formation of wildtype, mutants, and complemented mutants in yeast minimal medium (YMM medium) under light microscope at 28°C for 4 days. A= *flgC* mutant, B= *fliC* mutant, C=*fliD* mutant, D=*xagP* mutant, E=*fliC&fliD* mutant, F=*fliD&fliC* mutant, G=*fliD&xagP* mutant, H=pBBR-FIGC, I=pBBR-FLIC, J=pBBR-FLID, K=pPZP-XAGP, L=pPZP-FLIC&FLID, M=pPPZP-FLID&FLIC, N=pPZP-FLID&XAGP, and O=wildtype.

6. Flagellin protein analysis

The all of the mutants were examined for flagellin production visualized as a major 34.5-kDa band (Figure 13). Mutations that abolished flagellum biosynthesis also eliminated the activity of secreted proteins mentioned above. The results indicate that the FliC and FliD have roles in transport of proteins to the extracellular environment and that genes *flgC* and *fliC* are required for flagellin secretion; the *fliD* mutant secreted flagellin similar to wildtype. The results suggest that, *flgC* and *fliC* function in secretion and co-regulate expression of flagellin. The results also suggest the flagella regulon in Xag influences bacterial-host interactions in a manner independent of motility. Some of the effectors and proteins secreted by the flagella export apparatus could be subunits of the flagellum secreted by the bacterium during growth under laboratory conditions, as is the case for flagellin (Aizawa, 1996). Other proteins exported by flagella system include those that form the hook and the filament components (Aizawa, 1996). In addition, *flgC, fliC, fliD,* and *fliC&fliD* mutants showed reduction of all exoenzymes tested. The mutant strains *xagP* and *fliD&xagP*, completely lost pectolytic activity, whereas *flgC, fliC,* and *fliD* expressed reduced pectolytic activity. In this study, evidence is provided to indicate the flagellum export apparatus functions as a secretion system for transport of several extracellular proteins from Xag. Some of the exported proteins included flagellin and virulence factors exported to the outer surface of the cell (Young *et al.*, 1999). In addition, some proteins comprising the classical type III secretory apparatus show amino acid sequence similar to flagella structural proteins (Macnab, 2003). Moreover, the components of the flagella apparatus participate in the export of virulence determinants in several pathogens. Our work would suggest the export of virulence-associated proteins by the flagella export apparatus may be a general mechanism for the transport of proteins that influence bacterial-host interactions.

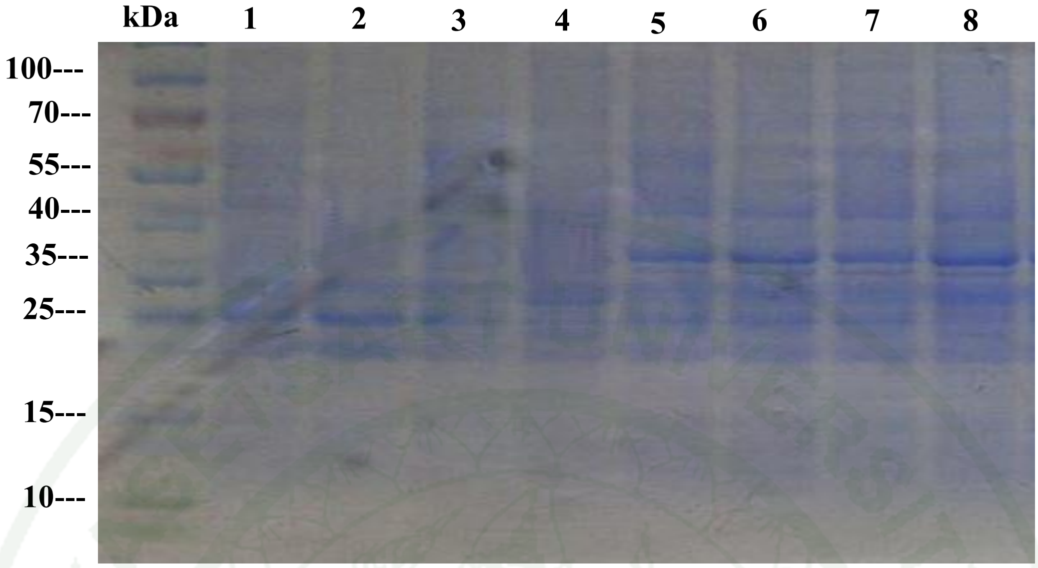

Figure 13 The 34.5 kDa of flagellin protein of *Xanthomonas axonopodis* pv. *glycines* 12-2 and their mutant were purified by SDS-PAGE, lane 1=*flgC* mutant, 2=*fliC* mutant, 3=*fliC* &*fliD* mutant, 4= *fliD&fliC* mutant, 5=*fliD* mutant, 6=*xagP* mutant, 7=*fliD* and *xagP* mutant, and 8=wildtype strain 12-2.

7. Pathogenicity test on soybean

The *flgC, fliC&fliD,* and *fliD&fliC* mutants completely lost pathogenesis, but *fliC, fliD, xagP,* and *fliD&xagP* reduced disease severity with 72.6, 95.6, and 98.1% respectively at 10 days after inoculation on soybean cv. Spencer, respectively (Table 6). In contrast, wildtype and complemented strains; *flgC* (pBBR-FLGC), *fliC* (pPZP -FLIC), *fliD* (pBBR-FLID), *xagP* (pPZP-XAGP), fliC&fliD (pPZP-FLIC&FLID), fliD&fliC (pPZP-FLID&FLIC), and fliD&xagP (pPZP-FLID&XAGP) gave reduced disease severiy of 22.5, 24.7, 21.0, 27.4, 20.4, 20.8, and 21.9 % on soybean cv. Spencer, respectively. Lesion expansion of mutants, *flgC, fliC, fliD, xagP, fliC&fliD, fliD&fliC,* and *fliD&xagP* were significantly smaller than wildtype with 0.5, 0.5, 0.8, 0.5, 0.5, 0.5, 0.5, and 0.5 cm, respectively (Table 6 and Figure 14). Number of pustule induction by wildtype was the highest with 8.4 pustule/ lesion whereas *fliC* mutant and double mutants (*fliC&fliD, fliD&fliC, fliD&xagP*) not showed the pustule observed at 10 days after infiltration. All the complemented strains of single and double mutants were showed the pathogenicity phenotype similar with wildtype strain

12-2 (Table 6). Haiko and Wikström (2013) reviewed in many bacterial species, the flagellum is an acknowledged virulence factor, and non-flagellated strains have in several cases been observed to be less virulent. The flagellum can act directly as an adhesin, as detailed in Sections 3 and 4, but can also affect virulence by other means. Motility towards a host cell is a prerequisite for adhesion and invasion, and flagella can play an essential role in colonization by facilitating bacterial motility even if the flagella do not directly participate in the adhesion or invasion. Flagella can also contribute to virulence by regulating the expression of other virulence factors (Prüss *et al.,* 2006) and, as discussed below, the flagellum in some cases affects virulence in more than one manner (Haiko and Wikström, 2013).

Adhesion of bacteria to plant surfaces Majority of bacteria do not require adhesion mechanisms except when they are moving through the xylem and phloem (Cao *et al.,* 2001). However, the crown gall bacterium Agrobacterium requires attachment to plant surface as the first step in the transport of T-DNA and to develop disease symptoms. The attachment needs three components; a glucan molecule, which requires three genes for its synthesis and export, genes for the production of cellulose and the att region of the bacteria genome that contains several genes for adhesion (Rodriguez-Navarro *et al.,* 2007). Additionally, Agrobacterium also has more other genes with homology to genes of mammalian pathogens for adhesions and for pilus biosynthesis (Agrios, 2005). Several other plant pathogenic bacteria also contain genes that encode proteins to be occupied in attachment and aggregation. R. solanacearum, anthomonas, Pseudomonas and Xylella have as many as 35 genes homologous to type IV pili genes, which are nvolved in cell to cell aggregation and protection from environmental stress in Xanthomonas and Pseudomonas, whereas type IV pili are essential in Xylella for the establishment of an aggregated bacterial population in the unstable environment of the xylem by adhering to the vessels in connection to components such as polysaccharides (Romantschuk, 1992).

Interestingly, the nonmotile single mutant *flgC, fliC* and double mutants including *fliC&fliD, fliD&fliC,* and *fliD&xagP* were reduced disease severity found on Spencer cultivars seem like they were lost pathogenesis and were nonpathogenic strains (Figure 14). Lesion expansion of single mutant; *fliC, xagP* and double mutant (*fliC&fliD, fliD&fliC,* and *fliD&xagP*) were significantly smaller than wildtype (Table 6). In addition, the lesion of *flgC, fliC, xagP,* and double mutant (*fliC&fliD, fliD&fliC,* and *fliD&xagP*) showed the necrosis region seem like HR at 48h after inoculation (Figure 15). Moreover, the pustules were occurred surrounding the infiltrated zone of wildtype and mutants. Number of pustule induction by wildtype was the highest with 8.4 pustule/ lesion whereas *fliC* mutant and double mutant not showed the pustule observed at 10 days after infiltration. The complemented strains of single mutant; *fliC*, *fliD*, *XagP,* and double mutant were showed the pathogenicity phenotype similar with wildtype (Table 6). Also, wildtype, mutants *fliC* and *fliD*, and all complemented strains induced HR on tobacco (*N. tabacum* cv. Xanthi, *N. glauca*, and *N. rustica*), chili, and tomato within 48 h except mutant *xagP* and double mutant (*fliD&xagP*) could not induced HR on nonhost plant (Table 8).

Table 6 Lesion size, disease severity, and number of pustule induction for wildtype, and flagella associated gene mutants, and complemented mutants on cultivar Spencer[1/]

Bacterial strain	**Lesion mean diameter (cm)[2/]**	**Disease reduction (%)[3/]**	**Number of pustule induction (pustule)[4/]**
flgC mutant	0.5a	100.0a	0.0a
fliC mutant	0.5a	96.8a	0.0a
fliD mutant	0.8b	72.6b	0.0a
xagP mutant	0.5a	95.6a	4.7b
Double mutant (*fliC&fliD*)	0.5a	100.0a	0.0a
Double mutant (*fliD&fliC*)	0.5a	100.0a	0.0a
Double mutant (*fliD&xagP*)	0.5a	98.1a	0.0a
pBBR-FLGC	0.5b	22.5c	8.2d
pBBR-FLIC	0.8b	24.7c	7.6d
pBBR-FLID	0.8b	21.0c	8.1d
pPZP-XAGP	0.8b	27.4c	6.4cd
pPZP-FLIC&FLID	0.8b	20.4c	6.1cd
pPZP-FLID&FLIC	0.8b	20.8c	6.5cd
pPZP-FLID&XAGP	0.8b	21.9c	8.2d
Wildtype (strain 12-2)	0.9b	0d	8.4d

[1/] Numbers followed by different letters show significance at $P \leq 0.05$.

[2/] Lesion mean diameter with 0.5 cm showed the necrotic symptom after 48 h infiltrated.

[3/] Disease severity was evaluated as described in text. For each strain at least three leaves, collected from the top, middle, and basal portion of three plants, were evaluated.

[4/] Number of pustules was calculated by total pustule surrounding the infiltration zone 10 days after infiltration.

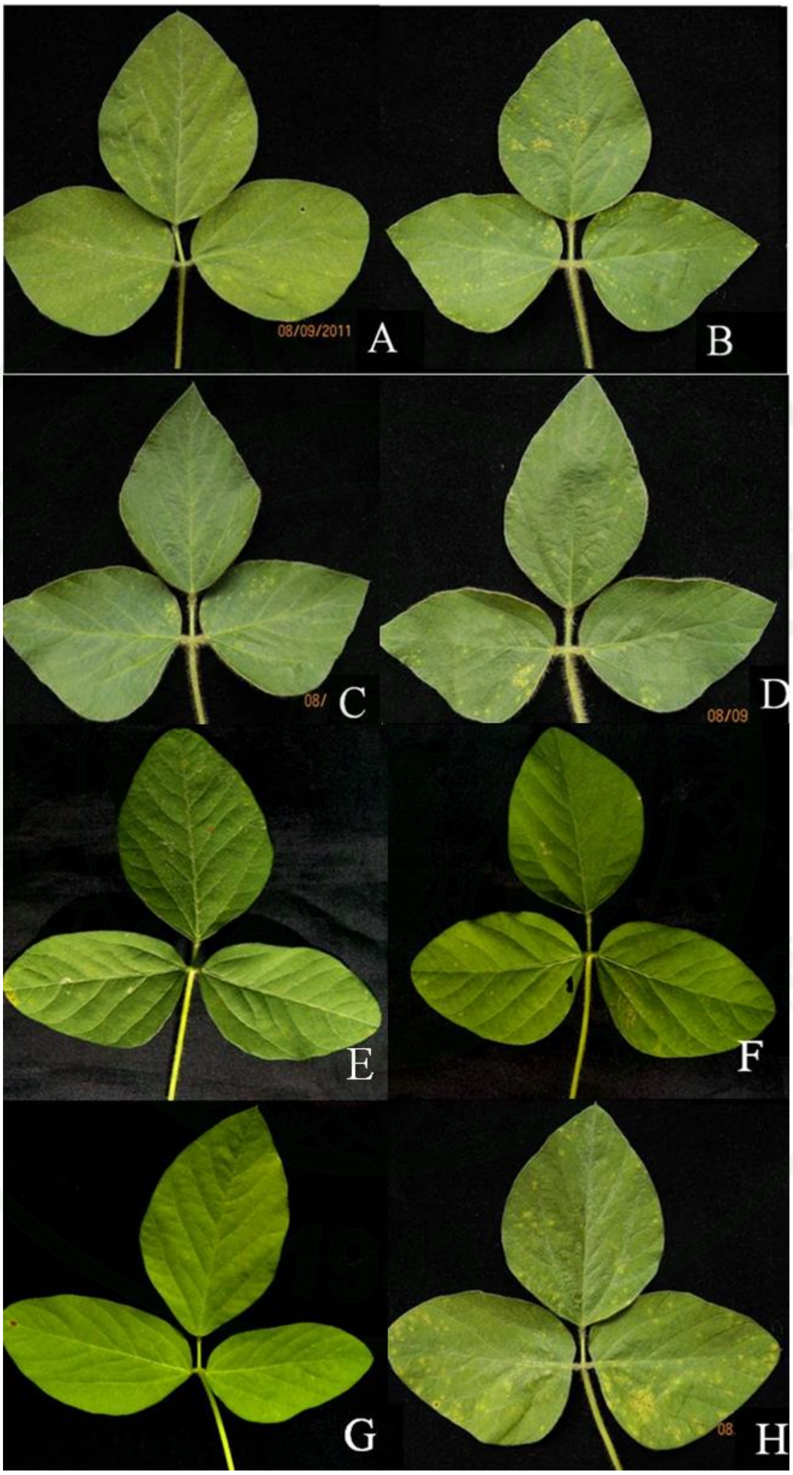

Figure 14 Soybean leaves were sprayed with mutants; *fliC* (A), *fliD* (B), *xagP* (C), *fliD&xagP* (D), *fliC&fliD* (E), and *fliD&fliC* (F) and *flgC* (G) compared to wildtype (H). They were photographed 10 days after inoculation.

8. Hypersensitive response induction

To evaluate the effect of flagella genes in virulence of *X. axonopodis* pv. *glycines*, we infiltrated host soybean leaves with bacterial suspension at 1x10^8CFU/ml. The *fliC*, *xagP*, and *fliD&xagP* mutants shown delayed disease symptom which showed the symptom like HR within 48 h after inoculation, a distinct chlorosis began to spread from the infiltration site, and necrosis appeared at the center of the inoculated area. After 7 days after inoculation appeared the narrow hallow yellow around the inoculated area. Whereas *fliD* and all complement showed caused typical progressive symptoms with hallow yellow around the inoculated area as wildtype strain 12-2 within 48 h after inoculation (Table 7 and Figure 15). Interesting, the *flgC, fliC&fliD* and *fliD&fliC* mutants showed the HR on soybean cv. Spencer within 48 h after inoculation. On the other hand, inoculation to all mutants and complements compared with wildtype on the difference three species of tobacco. All of bacterial strain caused the HR symptom with in 48 h, except *xagP* and *fliD&xagP* mutants were not cause the HR on tobacco plant (Table 9), Also, of bacterial strain can caused HR on tomato and chili (Table 7 and Figure 16, 17).

Table 7 Efficacy of *Xanthomonas axonopodis* pv. *glycines* mutants and *X. axonopodis* pv. *glycines* (wildtype) 12-2 for hypersensitive response on host and nonhost at 48 h after infiltrated

Bacterial strains	**HR induction** [1/]						
	Host		**Nonhost**				
	William 82	**Spencer**	***N. glauca***	***N. tabacum***	***N. rustica***	**Tomato**	**Chili**
flgC mutant	+	+	+	+	+	+	+
fliC mutant	+	D	+	+	+	+	+
fliD mutant	+	D	+	+	+	+	+
xagP mutant	+	D	-	-	-	+	+
Double mutant (*fliC*&*fliD*)	+	+	+	+	+	+	+
Double mutant (*fliD*&*fliC*)	+	+	+	+	+	+	+
Double mutant (*fliD*&*xagP*)	+	D	-	-	-	+	+
complemented *flgC* (pBBR-*flgC*)	+	D	+	+	+	+	+
complemented *fliC* (pBBR-*fliC*)	+	D	+	+	+	+	+
complemented *fliD* (pBBR-*fliD*)	+	D	+	+	+	+	+
complemented *xagP* (pPZP-XAGP)	+	D	+	+	+	+	+
complemented of double mutant (pPZP-FLID&XAGP)	+	D	+	+	+	+	+
complemented of double mutant (pPZP-FLIC&FLID)	+	D	+	+	+	+	+

Table 7 (Continued)

Bacterial strains	**HR induction** [1]						
	Host		**Nonhost**				
	William 82	**Spencer**	***N. glauca***	***N. tabacum***	***N. rustica***	**Tomato**	**Chili**
Wildtype (strain 12-2)	+	D	+	+	+	+	+
ddH_2O	-	-	-	-	-	-	-

[1] HR = hypersensitive response; + = positive reaction, - = negative reaction, D=disease symptom, infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h post inoculation. The negative response was no visible necrosis. The positive reaction was complete collapse and necrosis of the entire infiltrated area. The disease was necrosis symptom with hallow around lesion.

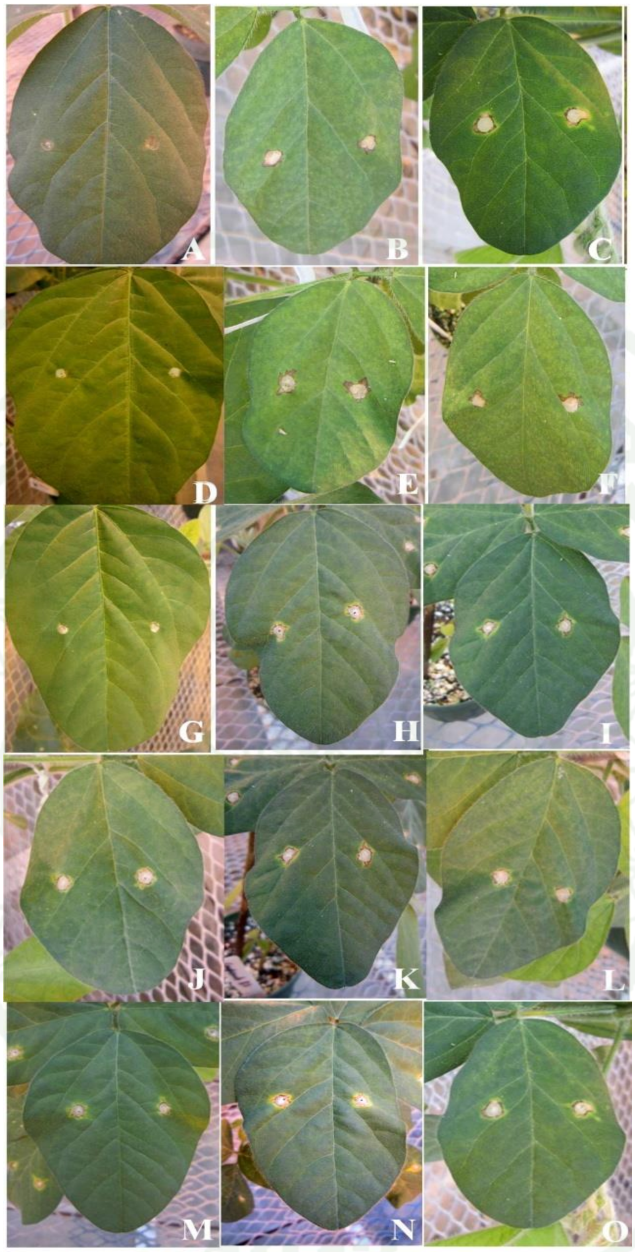

Figure 15 The hypersensitive response (HR) was induction on soybean. Soybean leaves were infiltrated with *X. axonopodis* pv. *glycines* 12-2 and mutant strains; A=*flgC* mutant, B= *fliC* mutant, C=*fliD* mutant, D=*xagP*, E=*fliC&fliD*, F=*fliD& fliC*, G=*fliD&xagP* and complemented strains; H= complement *flgC* (pBBR-FLGC), I=complement *fliC* (pBBR-FLIC), J=complement *fliD* (pBBR-FLID), K=complement *xagP* (pPZP-XAGP), L=complemented of double mutant (pPZP-FLIC& FLID), M=complemented of double mutant (pPZP-FLIC& FLID), N=complemented of double mutant (pPZP-FLID& XAGP), and O=wildtype strain 12-2 after 48 incubated.

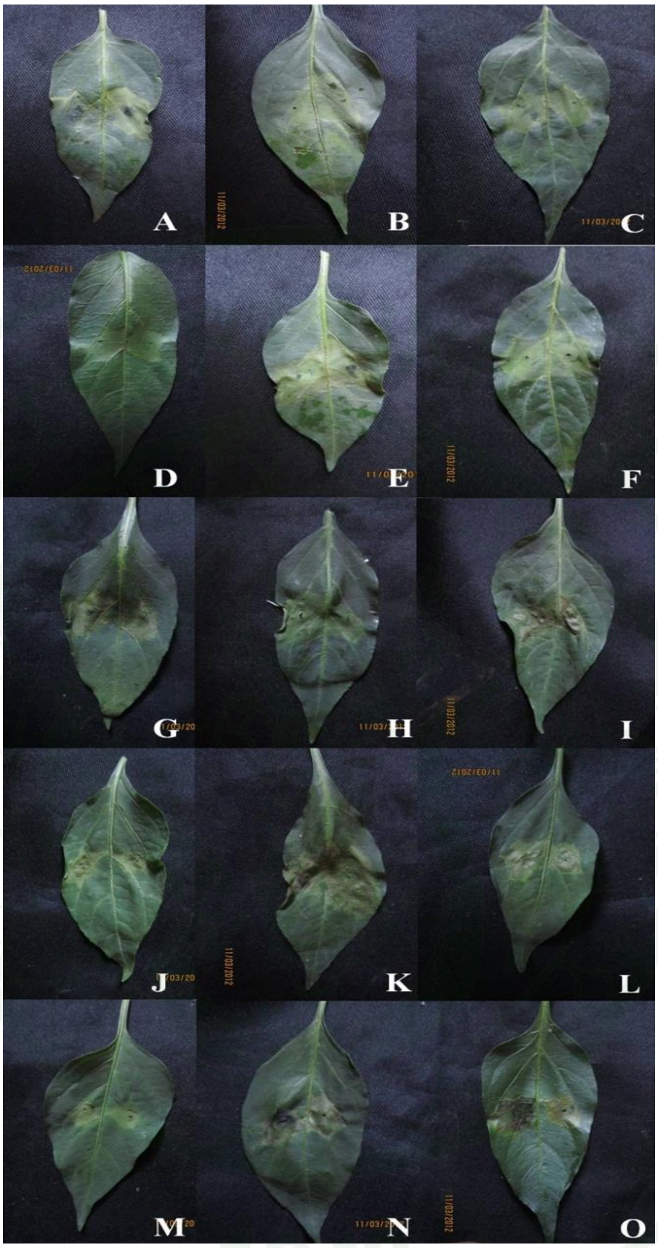

Figure 16 The hypersensitive response (HR) was induction on chili. Chili leaves were infiltrated with *X. axonopodis* pv. *glycines* 12-2 and mutant strains; A=*flgC* mutant, B= *fliC* mutant, C=*fliD* mutant, D=*xagP*, E=*fliC&fliD*, F=*fliD& fliC*, G=*fliD*&*xagP* and complemented strains; H= complement *flgC* (pBBR-FLGC), I=complement *fliC* (pBBR-FLIC), J=complement *fliD* (pBBR-FLID), K=complement *xagP* (pPZP-XAGP), L=complemented of double mutant (pPZP-FLIC& FLID), M=complemented of double mutant (pPZP-FLIC& FLID), N=complemented of double mutant (pPZP-FLID& XAGP), and O=wildtype strain 12-2 after 48 incubated.

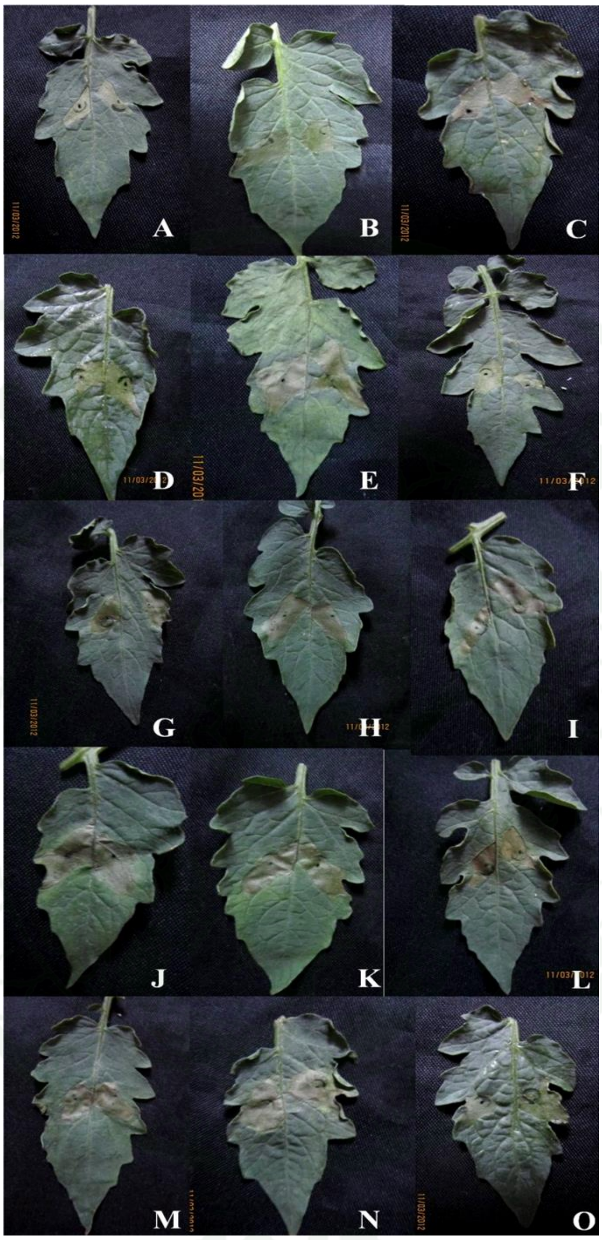

Figure 17 The hypersensitive response (HR) was induction on tomato. Tomato leaves were infiltrated with *X. axonopodis* pv. *glycines* 12-2 and mutant strains; A=*flgC* mutant, B= *fliC* mutant, C=*fliD* mutant, D=*xagP*, E=*fliC&fliD,* F=*fliD& fliC,* G=*fliD&xagP* and complemented strains; H= complement *flgC* (pBBR-FLGC), I=complement *fliC* (pBBR-FLIC), J=complement *fliD* (pBBR-FLID), K=complement *xagP* (pPZP-XAGP), L=complemented of double mutant (pPZP-FLIC& FLID), M=complemented of double mutant (pPZP-FLIC& FLID), N=complemented of double mutant (pPZP-FLID& XAGP), and O=wildtype strain 12-2 after 48 incubated.

9. Bacterial growth on soybean

The populations of *flgC, fliC, fliC&fliD* and *fliD&xagP* mutants following infiltration into soybean cv. Spencer were significantly less (P≤0.05) as compared to wildtype (Figure 18). The *fliD* mutant and all complemented mutants behaved the same as wild type on soybean cv. Spencer leaves up to 7 days after infiltration (data not shown). These results show a necessary role of flagella genes and *xagP* in pathogenicity. The flagellum is an exquisitely complex nanomachine that is the primary means for motility in many bacteria. Given that motility plays a vital role in important microbial processes such as chemotaxis, host colonization, and biofilm formation (Tsang and Hoover, 2014). The flagella gene mutant in *X. axonopodis* pv. *glycines* 12-2 were lost motility due bacteria to cannot respond to chemical and light gradients via chemotaxis and phototaxis, respectively.

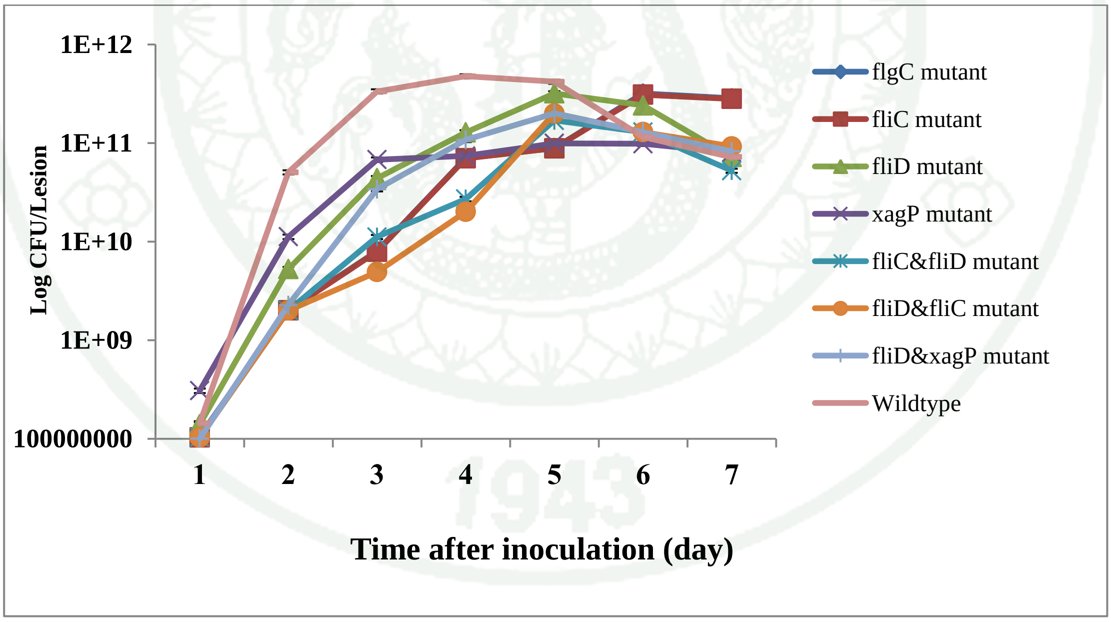

Figure 18 Time course of bacterial growth in soybean leaves Spencer. Leaves were sampled daily up to 7 days after infiltration. Data represents means from three repetitions, and vertical bars represents standard errors.

The results we suggests that the presence or absence of flagella is important to bacterial survival and growth. Also, Haiko and Wikström (2013) thus the bacterial flagellum affects bacterial virulence in various ways, i.e., by providing motility towards host targets, promoting early biofilm formation and thus bacterial survival, secreting virulence factors, triggering the adaptive and innate immune defense, and by promoting adherence and invasion.

10. The role of *rpoE* and *rpoN* regulated pathogenicity in *X. axonopodis* pv. *glycines* 12-2

Functional characterization of two genes *rpoE* and *rpoN* necessary for the full virulence of *X. axonopodis* pv. *glycines* 12-2 in relation to the expression of *xagP* and flagella genes (*flgC*, *fliC*, and *fliD*) revealed their contributions to bacterial growth and motility, biofilm formation, extracellular enzyme activity, diseased symptom and HR induction, and the relatedness between *rpoE* and *rpoN* as the following details.

10.1 Growth and motility

The *xagrpoE* and *xagrpoN* mutants were compared to the wiltype *in vivo* and *in vitro*. Colony appearance and morphology of two mutants on NGA were similar to wildtype strain 12-2. The *xagrpoE* mutant showed lower growth rate than that of the wildtype and of *xagrpoN* (Figure 19) when spray inoculation was conducted on soybean leaf surface under greenhouse conditions. The bacterial population of both *xagrpoE* and *xagrpoN* was significantly lower than the wildtype at 3, 4, and 5 day after inoculation. The *xagrpoN* mutant appeared to be rescued its growth. The results indicates that direct or indirect gene expression for epiphytic fitness of *X. axonopodis* pv. *glycines* may be under more control by *xagrpoE* than *xagrpoN*. The complementary strains showed similar growth rated to the wildtype (Figure 19).

The effect of mutation on motility was carried in 0.4% agar media. The motility assay in figure 20 and 21 showed a large number of wildtype cells that gave rose to localized confluent growth at the center of the agar surface. Both mutant strains were impaired in motility that *xagrpoN* showed slower movement than *xagrpoE* suggesting *xagrpoN* regulated the flagella gene expression. The *xagrpoN* mutant was unable to coalesce or co-migrate between adjacent colonies therefore, the small aggregation was formed.

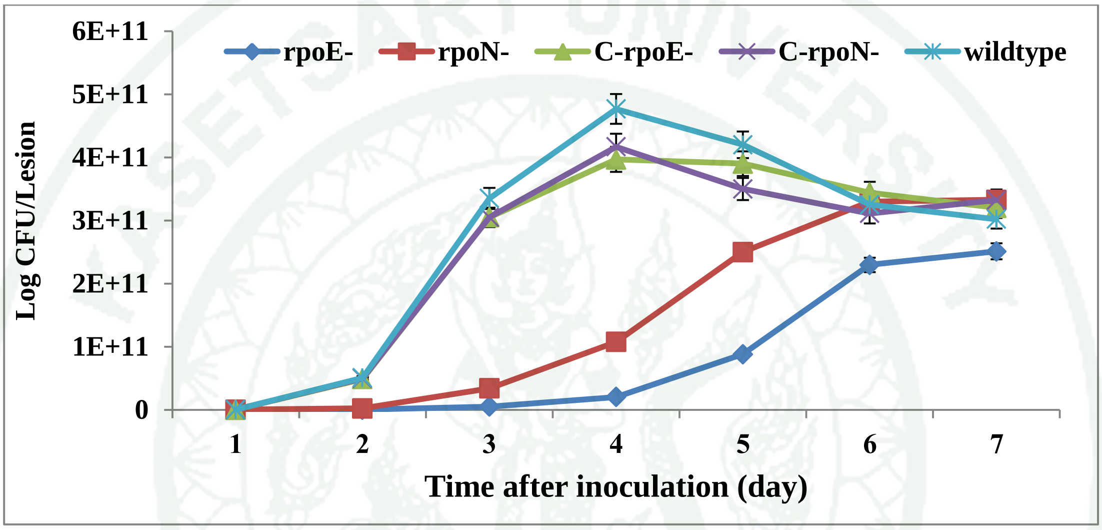

Figure 19 Time course of bacterial growth in soybean leaves cv. Spencer. Leaves were sampled daily up to 7 days after infiltration. Data represents means from three repetitions, and vertical bars represents standard errors.

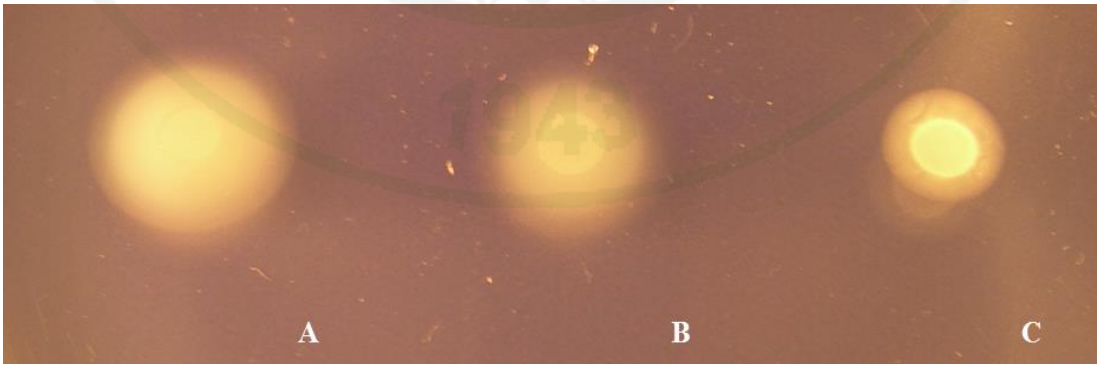

Figure 20 Motility of *xagrpoE* (B) and *xagrpoN* (C) mutant strains NGA (0.4% agar) compared with wildtype 12-2 (A) after incubated at 28^{o}C for 48 h.

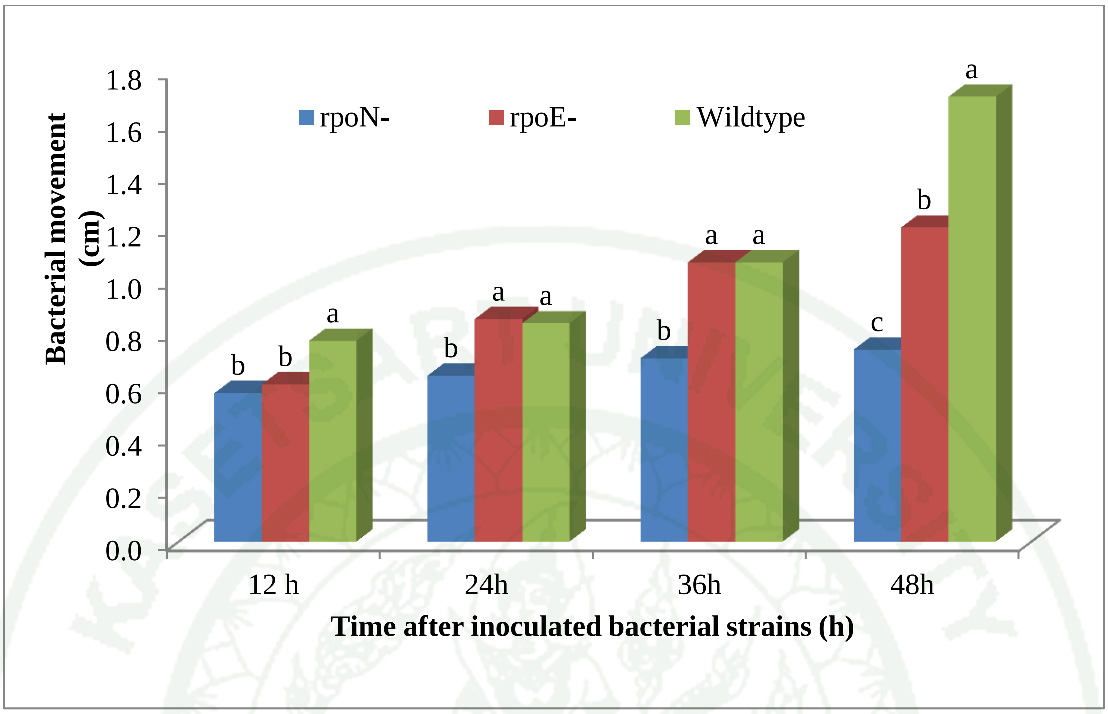

Figure 21 Kinetics of *xagrpoE* and *xagrpoN* mutant strains movement on NGA (0.4% agar) compared with wildtype 12-2 after incubated at 28°C for 48 h.

10.2 Biofilm formation

To examine the roles of *rpoE* and *rpoN* in biofilm formation the ability of 12-2 and mutants to develop biofilms were assessed in 96-well polystyrene plates and culture in YMM broth. When 12-2 was cultured in YMM it formed visible biofilms in wells after continuous agitation. In contrast, *rpoE* and *rpoN* were exhibited significantly reduced biofilms formation when compared with wildtype (Figure 22). These results indicate that specific *rpoE* and *rpoN* mutants play significant roles in biofilm formation. The *rpoE* and *rpoN* appeared to be typically associated with the microorganism to colonize surfaces, and in most cases these microorganism do not invade cells such as *X. axonopodis* pv. *glycines*, it stabilizes in intercellular cell. Reduced biofilm formation in the mutants could be due to a weakened outer membrane that resulting lost pathogenicity (Sandkvist *et al.*, 1997). Also, genes affecting twitching motility have been shown to be important for biofilm formation and then infection by *P. aeruginosa* (Comolli *et al.*, 1999; Argenio *et al.*, 2001). *P.*

aeruginosa is also an important model (O'Toole and Kolter, 1998) and which appears to be involved in disease infections (Whitchurch *et al.*, 2002). Moreover, *rpoN* obviously controls the quorum sensing system via a positive regulation of RhlI, the auto-inducer synthase responsible for the synthesis of N-butyryl-L-homoserine lactone (Thompson *et al.*, 2003). In *rpoN* mutants, it was shown that the expression of the *lasR* and *lasI* genes was elevated at low cell densities and growth (Heurlier *et al.*, 2003).

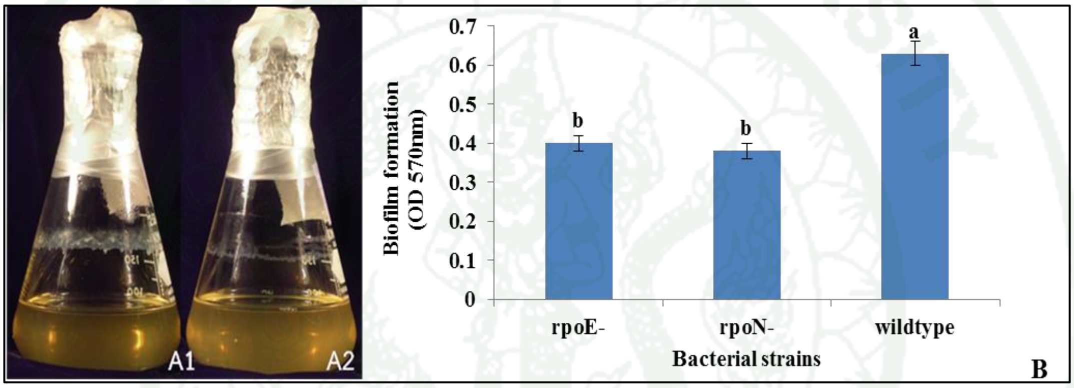

Figure 22 Bioflim formation of *X. axonopodis*pv. *glycines* 12-2 (A1) and *rpoE* mutant strain (A2). Bacterial strains were grown in nutrient agar. After incubation at 28^{0}C for 10 days, biofilm formation was measured by spectrophotometer at 570 nm absorbance (B).

10.3 Assay of extracellular enzyme secretion

The *xagrpoE* and *xagrpoN* affected extracellular enzyme activity were not different. They all reduced synthesis and secretion of alpha-amylase, cellulase, endoglucanase, pectate lyases, protease, and polygalacturonase activity. Strains 12-2 and mutants; *rpoE* and *rpoN* were compared for expression of different exoenzymes (Figure 23), suggesting that activity of both sigma factors; *xagrpoE* and *xagrpoN* are necessary for full virulence in *X. axonopodis* pv. *glycines* 12-2.

10.4 Pathogenicity and HR assay

The *xagrpoE* and *xagrpoN* mutant showed reduced disease severity was measured at 10 days after inoculation on soybean cv. Spencer with 11.1 and 14.8% disease severity respectively, compared to wildtype (Figure 24). The mutant *xagrpoE*, *xagrpoN*, complemented mutants, and wildtype strains all showed typical progressive HR symptoms included a yellow halo around the inoculated within 48 h after inoculation (Figure 25). The hypersensitive response, or HR, is a form of cell death often associated with plant resistance to pathogen infection. Reactive oxygen intermediates and ion fluxes are proximal responses probably required for the HR. Apoptosis as defined in animal systems is, thus far, not a strict paradigm for the HR. The diversity observed in plant cell death morphologies suggests that there may be multiple pathways through which the HR can be triggered. Signals from pathogens appear to interfere with these pathways. HR may play in plants the same role as certain programmed cell deaths in animals with respect to restricting pathogen growth. In addition, the HR could regulate the defense responses of the plant in both local and distant tissues (Marutani *et al.*, 2005).

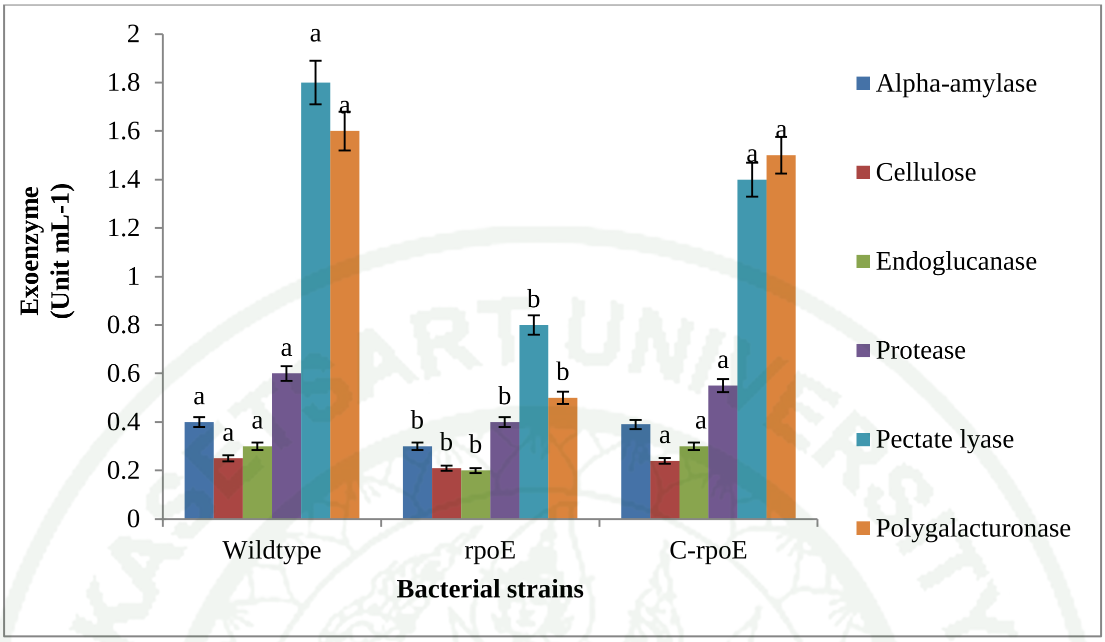

Figure 23 *RpoE* mutants exhibited reduced production of alpha-amylase, cellulose, endoglucanase, protease, and pectate lyase activities compared to the wildtype strain 12-2 and complementary strain.

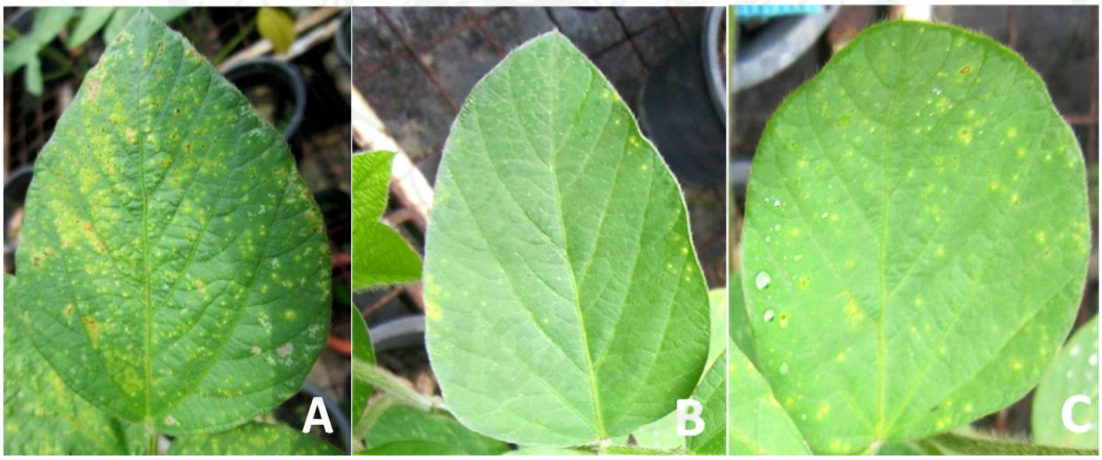

Figure 24 The disease severity was induction on soybean. Soybean leaves were sprayed with wildtype (B), *xagrpoE* mutant (B), and complementary strain (C). They were photographed 10 days after inoculation.

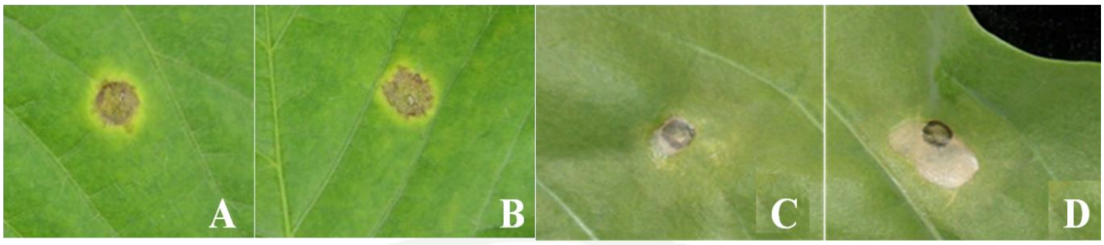

Figure 25 The hypersensitive response (HR) induced on host (soybean cv. Spencer) and nonhost (tobacco; *Nicotiana tabacum* cv. Xanthi). Soybean and tobacco leaves were infiltrated with *xagrpoE* mutant (A and C) compared with wildtype (B and D) after 48 h infiltration.

10.5 Analysis of gene expression

This experiment was conducted to determine the expressions of flagella (*flgC, fliC, fliD),* and pectate lyase (*xagP)* genes in *xagrpoE* and xag*rpoN* mutant strains. The results showed down-expression of *flgC, fliC,* and *xagP* in *xagrpoE* and xag*rpoN* except *fliD* gene in *xagrpoE* mutant that showed similar expression with wildtype (Figure 26).

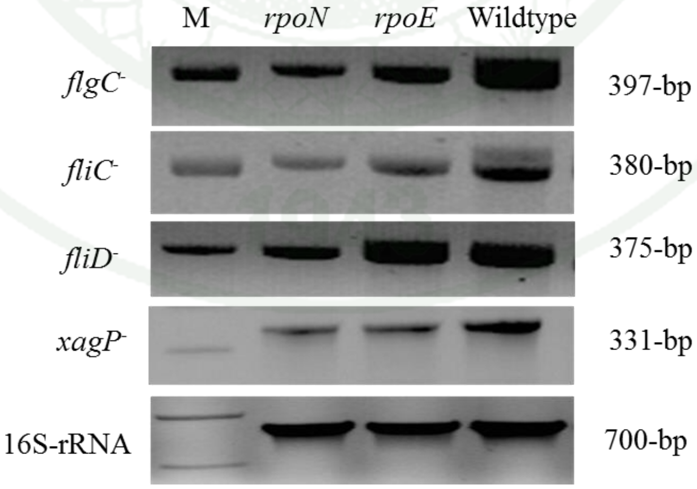

Figure 26 Gene expressions of *flgC, fliC, fliD,* and *xagP* genes which 16S-rRNA used as internal standards in *rpoE*$^-$ and *rpoN*$^-$ mutants compared with wildtype. Lane 1= 1 Kb marker, lane 2=*rpoN*$^-$, lane=*rpoE*$^-$, and lane 4= wildtype 12-2.

Analysis of flagella and pectate lyase genes expression in *rpoE* and *rpoN* mutants using NanoDrop analysis, we found that the rpoE have affaced to suppress expression *flgC, fliC, fliD* and *xagP* genes with 40.0, 6.67, 5.26, and 25.0 % respectively, whereas rpoN suppress these genes expression with 56.0, 33.3, 36.8, and 30.0% respectively when compared with wild type (Figure 27). This result correlated with *xagrpoE* and *xagrpoN* that have defected extracellular enzymes secretion especially pectate lyase. They were influent on virulence with 11.1 and 14.8% increased disease severity on Spencer compared to wildtype. The results implied that the *xagrpoE* and *xagrpoN* genes regulated expression of flagella and pectase lyase genes. The *rpoE* regulated gene expression of the extracellular enzymes especially pectase lyase, whereas *rpoN* regulated the flagella genes (*fliC*) of Xag 12-2 expression.

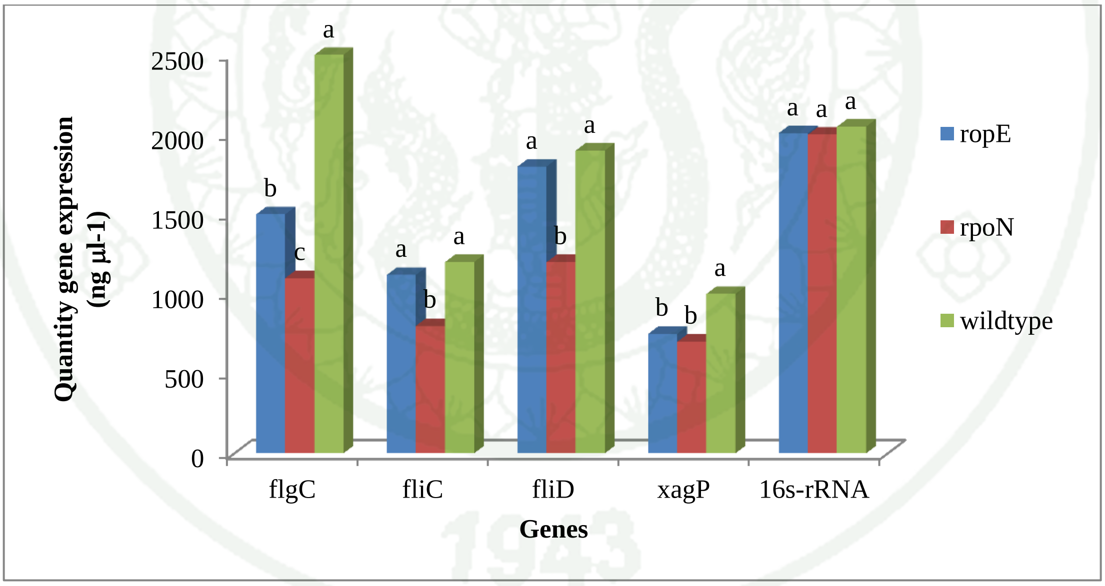

Figure 27 Expressions of *flgC*, *fliC*, *fliD*, *xagP*, and 16S-rRNA genes, as internal standards in *rpoE* and *rpoN* mutants compared to wildtype was investigated by RT-PCR and NanoDrop analysis that mention in Materials and Methods.

Discussion

The 4-flagella mutants; *flgC, fliC, fliC&fliD,* and *fliD&fliC* were completely lost swimming and *fliD* and *fliD&xagP* mutant motility were reduce. Because *fliC* encodes the flagellin protein itself, the *fliC* mutant did not produce flagellin. On the other hand, *fliD* encodes the hook associated protein2 (HAP2)-capping protein that functions to polymerize monomer flagellin to form the filament at the top of the flagellum (Shimizu *et al.*, 2003). Flagellar motility is an ancient, conserved mechanism that may have evolved from the same ancestor as T3SS (Aizawa, 2001). Thus, the *fliD* mutant did not form a flagellum but secreted a large amount of monomer flagellin into the culture medium (Shimizu *et al.*, 2003). This motility facilitates bacterial migration towards less hostile environments. In non-motile bacteria, however, the presence of flagella would be evolutionarily redundant and energetically expensive, unless the proteins played a role in another mechanism involving bacterial replication or survival (*Stone et al.*, 2010). When we inoculated soybean leaves with *flgC, fliC, fliC&fliD,* and *fliD&fliC* mutants, the mutants failed to spread disease symptoms. Moreover, the deletion of each single flagella gene (*flgC* or *fliC* or *fliD*) mutants resulted in reduced virulence on soybean plant. The responses of soybean leaves to inoculation with the flagella-defective mutants were distinctly different. The *flgC, fliC, fliC&fliD, fliD&fliC* and *fliD&xagP* mutants caused limited and delayed symptoms, whereas the *fliD* and mutant caused reduced symptoms when compared with wildtype. As shown in this study, flagella seem to be important for the virulence of *X. axonopodis* pv. *glycines* strain 12-2 on host soybean plants. Based on the results obtained in this study, we discuss the correlations between motility, flagellin production, and virulence of *X. axonopodis* pv. *glycines* on host soybean plants. FliC, as a flagellar filament structural protein, is hypothesized to be involved in the pathogenesis of infection (He *et al.*, 2012).

In addition to regulation of synthesis of pathogenicity factors, the result indicated flagella genes; *flgC, fliC* and *fliD* and *xagP* genes regulated virulence factor as extracellular enzymes (alpha-amylase, cellulose, endoglucanase, pectase lyase, and protease). These virulence factors were regulated by flagella genes of *X. axonopodis* pv. *glycines* 12-2, except pectase lysae were regulated by *xagP* gene, to help the pathogen infect the hosts. Furthermore, one enzyme has established role in virulence in *X. axonopodis* pv. *glycines* is pectase lyase. *XagP* and *fliD&xagP* mutants of *X. axonopodis* pv. *glycines* could regulate the pectase lyase synthesis. These enzymes degrade polygalacturonates and other pectic components in plant cell walls are caused of tissue maceration (Gomathi and Gnanamanickam, 2004), in *X. axonopodis* pv. *glycines*, *xagP* gene which involved pectase lyase synthesis (Kaewnum *et al*., 2006). We expect that flagella genes is involved or participated with *xagP* for pectase lyase synthesis.

Pectolytic and cellulolytic enzymes that degraded components of the plant cell wall are the main virulence factors; they are predominantly secreted via type II pathway. Endo-glucanases (carboxylmethyl cellulases) that hydrolyze cellulose are abundantly produced by *Erwinia* spp. and *Xanthomonas* spp. Pectic polymers of the primary plant cell wall and middle lamella are cleaved at α-1,4-galacturonyl linkages by hydrolysis (polygalacturonases) or β-elimination (pectin and pectase lyases), whereas pectin methylesterases remove methoxyl groups on galacturonate residues of the chain (Barras *et al*., 1994). Due to the abundance of the cell wall structural components and to their importance for its integrity, the enzymatic attack leads to severe maceration of plant tissue and finally killing of plant cells. In contrast to *Erwinia* spp. and vascular pathogenic *Xanthomonas* spp., *Pseudomonas syringae* and many *Xanthomonas* spp. are host-specific, biotrophic pathogens that multiply in the mesophyll. The latter pathogens cause foliar chlorosis and the late necrosis rather than rotting and show a weaker and variable pectolytic activity. Genes encoding pectic enzymes have been identified in some strains, but their contribution to the pathogenicity of bitrophic bacteria is not clear (Boch and Bonas, 2001).

Navarro and coworker (2010) reported that bacteria are exposed to many stressful conditions in nature, including nutrient starvation, which can limit their growth for long time periods. In laboratory conditions, starvation is mimicked by exhaustion of a growth-limiting element from the culture medium, most often the carbon source, and leads bacterial cells to transit from exponential growth to the so-called stationary phase, characterized by the absence of visible growth (Navarro *et al.,* 2010; Nyström, 2004). Bacteria have evolved a number of different strategies that make them able to survive these famine periods. Those strategies mainly rely on global reorganization of gene expression, resulting in a number of morphological, physiological and metabolic changes, some of which confer multiple stress resistance to the cells and thus improve their ability to survive on the long term (Navarro *et al.,* 2010). Among the regulators involved in this reprogramming are alternative sigma factors, which by associating with RNA polymerase (RNAP) change its specificity of promoter recognition. In exponential phase, the vegetative sigma factor is responsible for the transcription of housekeeping genes. At the onset of stationary phase, various signals and mechanisms make alternative sigma factors available for interacting with the core RNAP, thus allowing the holoenzyme to recognize new promoters and express new sets of genes. In *Escherichia coli*, the master general stress response regulator RpoS is the main sigma factor in charge of gene expression in stationary phase, as it controls the transcription of several hundreds of genes involved in functions as diverse as acquisition of multiple stress resistance, redirection of metabolism or structuration of the cell envelope (Hengge, 2011; Battesti *et al.*, 2011). Other alternative sigma factors, such as the heat shock sigma factor RpoH or the extracytoplasmic function (ECF) sigma factor RpoE are also active at the end of exponential phase and upon entry in stationary phase, respectively, and mainly control the expression of chaperones and proteases involved in folding and degradation of cytoplasmic and secreted proteins, respectively (Costanzo and Ades, 2006; Fredriksson *et al*., 2005; Wagner *et al.,* 2009).

The *flgC, fliC, fliC&fliD,* and *fliD&fliC* mutants were found to be nonmotile, whereas the *fliD* and double *fliD&xagP* mutants were reduced motility. The results indicate that only motility of bacteria is necessary for the formation of biofilm, but not the physical presence of flagella. We also raised the possibility that flagella-mediated motility may not be directly involved in pathogenicity but may be involved in the formation of a biofilm, a factor required for pathogenicity (Hossain and Tsuyunu, 2006). Recent molecular and genetic studies have identified several genes whose products are thought to be important for biofilm formation. Flagella or twitching-mediated motility has been reported to be important for free swimming cells of several bacterial species to initiate the formation of biofilms (O’Toole and Kolter, 1998; Pratt and Kolter, 1998; Watnick *et al.,* 1999). In addition, the production of EPS (Davies *et al.,* 1993; Boyd and Chakrabarty, 1995; Davies and Geesey, 1995; Yildiz and Schoolnik, 1999) and outer membrane proteins (Danese *et al.*, 2000) were also shown to be required for biofilm formation. The catabolite repressor control (Crc) protein, which acts as a positive factor to induce the expression of many genes including those for carbon and metabolism, was also reported to be necessary for biofilm formation in *P. aeruginosa* (O’Toole *et al.*, 2000).

Previously have been reported the biofilm formation is a major pathogenetic factor of *Staphylococcus epidermidis*. In *S. epidermidis* the alternative sigma factor sigma B was identified to regulate biofilm formation in *S. epidermidis* 1457. In *S. aureus* sigma B dependent regulation plays a minor role, whereas *sarA* (*Staphylococcus* accessory regulator) is an essential regulator. Therefore, we investigated the impact of sigma B on *sarA* transcription and biofilm formation in three independent *S. epidermidis* isolates. Mutants with dysfunctional sigma B displayed a strongly reduced biofilm formation, whereas in mutants with constitutive sigma B activity biofilm formation was increased. Transcriptional analysis revealed that *icaA* transcription was down-regulated in all sigma B negative mutants while *icaR* transcription was up-regulated. However, transcriptional differences varied between individual strains, indicating that additional sigma B-dependent regulators are involved in biofilm expression. Interestingly, despite the presence of a sigma B promoter beside two sigma A promoters no differences, or only minor ones, were observed in *sarA* transcription, indicating that sigma B-dependent *sarA* transcript has no influence on the phenotypic changes. The data observed in independent clinical *S. epidermidis* isolates suggests that, in contrast to *S. aureus*, regulation of biofilm formation by sigma B is a general feature in *S. epidermidis.* Additionally, we were able to demonstrate that the *sarA*- dependent regulation is not involved in this regulatory pathway (Jäger *et al.*, 2009).

Bacterial populations consisting of single or multiple species that are attached to a surface are commonly referred to as biofilms (Costerton, 1995; Costerton *et al.,* 1995, 1999; Geesey *et al.,* 1977). Bacteria in aquatic environments are often found in association with a solid surface rather than in a free-swimming phase (Costerton *et al.,* 1987). Attached bacteria may exist as a dispersed monolayer of cells on a surface in a microcolony or in a three-dimensional structure (Costerton *et al.,* 1995). Bacteria in biofilms are more resistant to adverse environmental conditions such as desiccation and extreme temperature (Zottola and Sasahara, 1994; Costerton *et al.,* 1995; Dewanti and Wong, 1995). Biofilm-associated bacteria also generally acquire resistance to antimicrobial agents (Hoyle and Costerton, 1991; Ichimiya *et al.,* 1994), and are thought to be protected from the host defense responses of animals and plants (Kharazmi, 1991; Leid *et al.,* 2002; Walker *et al.,* 2004). In this sense, the bacterial biofilm may be considered as an important virulent factor of several animal and plant pathogenic bacteria (Verhoef, 1997). Biofilm development is a multistep process. In general, its developmental pathway proceeds from the initial attachment and immobilization of bacteria to a solid surface, followed by the formation of the cell cluster or microcolony and ultimately by a three-dimensional architecture. The three-dimensional structure is created by pillars of bacteria surrounded by water channels that allow the influx of nutrients and the efflux of waste products (Costerton *et al.,* 1995).

Many microorganisms in the natural environment exist in multicellular aggregates generally escribed as biofilms, associated with solid surfaces and in intimate contact with other microbial cells. Cells adhere to surfaces and each other through a complex matrix comprising a variety of extracellular polymeric substances (EPS) including exopolysaccharides, proteins and DNA. Biofilm formation on plants is associated with symbiotic and pathogenic responses, but how plants regulate such associations is unclear. Certain bacteria in biofilm matrices have been found to induce plant growth and to protect plants from phytopathogens (a process termed biocontrol), whereas others are involved in pathogenesis. Bogino and coworker (2013) systematically describe the various components and mechanisms involved in bacterial biofilm formation and attachment to plant surfaces and the relationships of these mechanisms to bacterial activity and survival (Bogino *et al.*, 2013).

O'Toole *et al.* (2000) have been repoted that the biofilms can be defined as communities of microorganisms attached to a surface. It is clear that microorganisms undergo profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm bacteria and occur in response to a variety of environmental signals. Recent genetic and molecular approaches used to study bacterial and fungal biofilms have identified genes and regulatory circuits important for initial cell-surface interactions, biofilm maturation, and the return of biofilm microorganisms to a planktonic mode of growth. The planktonic-biofilm transition is a complex and highly regulated process (O'Toole *et al.*, 2000).

Biofilms can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces. Although mixed-species biofilms predominate in most environments, single-species biofilms exist in a variety of infections and on the surface of medical implants (Adal and Farr, 1996; Ascon-Cabrera *et al.*, 1995; Dickinson and Bisno, 1993). These single-species biofilms are the focus of most current research. *P. aeruginosa* has emerged as the most studied single-species, biofilm-forming gram-negative bacterium, although, as detailed in this review, among the gram-negative bacteria, *P. fluorescens*, *E. coli*, and *Vibrio cholerae* have also been studied in detail. The gram-positive biofilm-forming bacteria that have been studied include *Staphylococcus epidermidis*, *Staphylococcus aureus*, and the enterococci.

For secretion of proteins, special export systems are required to guide proteins through the physical barrier of membranes (Pugsley, 1993). As Gram-negative bacteria have two different kinds of membranes, the inner and outer membrane, the export system penetrates both membranes. Consequently, many genes are necessary for elaborating such a complicated system. Three types of export systems are currently well known (Pugsley, 1993). Recently, it has been shown that called type III export systems in many pathogenic bacteria play an essential role in pathogenicity or, more specially, virulence; often, one or more gene clusters called pathogenicity islands encode both virulence factors and structural components of the export apparatus through which the factors are secreted (Hueck, 1998; Lee, 1997).

In *S. typhimurium*, there are two kinds of type III secretion systems: one for flagella biogenesis and one for the export of virulence factors. The flagellum is an extracellular structure that grows distally, and the component proteins are exported through its central channel to the growing end. There is a hole (about 2 nm in diameter) running along in the centre of a flagella filament (Mimori *et al.,* 1995). The passage initiates at the basal body, where a gating mechanism at the cytoplasmic side selects flagella proteins only out of an overwhelmingly large number of other proteins. It should be noted that the flagella basal structure is engaged in protein secretion, before it works as the flagella motor later on.

The transportation of flagella proteins should not be considered as secretion in a general sense, because most of the proteins to be transported are not intentionally emitted into media or environment. But they are mistakenly emitted; there must be some leaks or spill-over during construction. For example, flagellin is secreted into media as a monomer in the absence of FliD, a cap protein, which helps flagellin to polymerize into flagella filaments (Ikeda *et al.,* 1996).

The bacterial flagellum is an intricate nanomachine, which is made of about 30 different proteins (Macnab, 2003). It rotates like a propeller to enable the cells to swim (Chevance and Hughes, 2008; Macnab, 2004). At the base of the flagellum a type III export apparatus is proposed to exist for the secretion of the external protein components. The type III secretion system, sometimes referred to as the needle complex, which is found in some pathogenic Gram-negative bacteria also uses a type III export apparatus. This highly homologous export apparatus is used to inject proteins for infection into Eukaryotic host cells, which is a major source of pathogenicity (Cornelis, 2006; Galán and Wolf-Watz, 2006). The proton motive force is the energy source for the export apparatus of both flagellar assembly and the needle complex, and in bacteria such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) it is the energy source for the rotation of the fully-formed flagellum (Macnab, 2003; Minamino and Namba, 2008; Paul *et al.*, 2008).

The bacterial flagella export apparatus consists of about five membrane proteins and three soluble proteins. The membrane proteins are highly conserved with a high sequence identity from bacterial species to species, and to the export apparatus of the needle complex (Blocker *et al.*, 2003). These proteins are called FlhA, FlhB, FliP, FliQ and FliR. They are essential for protein export. In the *Salmonella* flagellar export apparatus there is a less conserved and non-essential sixth membrane protein, FliO (Barker *et al.*, 2010). It has no homolog in the virulence-associated needle complexes and is absent from flagella systems of some bacteria (Macnab, 2004). The *Salmonella* flagella export system also contains three soluble proteins, FliH, FliI, and FliJ. FliI is an ATPase, which shows structural similarity to the α and β subunits of F_0F_1-ATP synthase (Minamino *et al.*, 2008). FliI is proposed to use the energy from ATP hydrolysis to enable flagella protein release into the pore of the secretion apparatus. For the experiment we found that the *X. axonopodis* pv. *glycines* 12-2 flagella export system also contains FlgC and FliC proteins. The export apparatus for virulence factors in *S. typhimurium* was identified as the needle complex (Kubori *et al.*, 1998). The needle complex looks similar to the flagella basal body by electron microscopy. Several genes from the two systems share common sequence features, suggesting that they might originate from a common ancestor.

Barras *et al.* (1994) have been reported the pectolytic and cellulolytic are predomainantly secreted via the type II pathway. The pectolytic and cellulolytic enzymes that degrade components of the plant cell wall are the main virulence factors. Endoglucanases (carboxymethyl cellulases) that hydrolyze cellulose are abundantly produced by *Xanthomonas* spp. Pectic polymers of primary plant cell wall and middle lamella are cleaved at α-1,4-polygalaturonyl linkages by hydrolysis (polygalacturonases) or β-elimination (pectin and pectate lyases), whereas pectin methylesturonases remove methoxy groups on galacturonate residues of the chain

(Boch and Bonas, 2001). For some bacteria the primary virulence characteristic is the coordinated production of large amounts of multiple secreted plant cell wall-degrading enzymes (PCWDEs), which leads to the breakdown of plant tissue (Toth *et al.*, 2003). The most important PCWDEs are the pectinases of *Erwinia carotovora* subsp. *carotovora*: primarily multiple isoforms of pectate lyase (Pel), together with isoforms of polygalacturonase (Peh), pectin methylesterase, and pectin lyase (Thomson *et al.*, 1999). We found that the *xagP* mutant resulted in loss pectate lyase production when compared to wildtype 12-2. Although Pels have been shown to play significant roles in pathogenesis of certain bacterial pathogens (Collmer and Keen, 1986), the *xagP* in Xag was found to be required for HR induction on several plant species (Kaewnum *et al.*, 2006). For the *T4P* mutant, Chuaboon *et al.* (2013) found that the entire mutant strain could produce the pectate lyase whereas could not fully secretion because of lack of the pili. Finally the soybean plant hardly appears the disease symptom. Mutants neither expressed haloes of HR nor scattered lesions over spray-inoculated leaves of disease development, but revealed pustule around the leaf margin at hydathode pores. We proposed the hypothesize that the *X. axonopodis* pv. *glycines* 12-2 can direct entry through hydrathode, where bacteria lack active mechanisms for gaining entry as lack twitching motility (*T4P* mutant), and plants similarly lack active mechanisms for preventing entry. However bacteria cannot directly penetrate the leaf epidermis and must enter leaf tissues through natural openings or wounds on the leaf surface. Stomata dominate in number among natural openings in the aerial part of the plant. Other natural openings include hydathodes in leaf margins, nectarthodes in flowers, and lenticels in stems and roots (Huang, 1986). However, recent evidence suggests that entry of bacteria into leaf tissue is more complex and dynamic than the simple act of swimming into the leaf through passive openings (Melotto *et al.*, 2006).

Interestingly, the nonmotile single mutant *fliC* and double mutants including *fliC& fliD, fliD&fliC,* and *fliD&xagP* were reduced disease severity found on Spencer cultivars (susceptible) seem like they were lost pathogenesis and were nonpathogenic strains. Lesion expansion of single mutant; *fliC, xagP* and double mutant (*fliC&fliD, fliD&fliC,* and *fliD&xagP*) were significantly smaller than wildtype. In addition, the lesion of *fliC, xagP,* and double mutant (*fliC&fliD, fliD&fliC,* and *fliD&xagP*) showed the necrosis region seems like HR at 10 days after inoculation and were no surrounding yellow halo after keep in the greenhouse for 1 month. Moreover, the pustules were occurred surrounding the infiltrated zone of wildtype and mutants. Number of pustule induction by wildtype was the highest with 30.26 pustule/ lesion whereas *fliC* mutant and double mutant (*fliC&fliD* and *fliD&xagP*) not showed the pustule observed at 10 days after infiltration. The complemented strains of single mutant; *fliC*, *fliD*, *XagP*, and double mutant (*fliD* and *xagP*) were showed the pathogenicity phenotype similar with wildtype. Also, wildtype, mutant's *fliC* and *fliD*, and all complemented strains induced HR on three species of tobacco (*N. tabacum* cv. Xanthi, *N. glauca*, and *N. rustica*) within 48 h except mutant *xagP* and double mutant (*fliD&xagP*).

Taguchi *et al.* (2003) have been reported that the flagellin, a major component of the flagellum filament of *Pseudomonas syringae* pvs. *tabaci*, *glycinea*, and *tomato*, was identified as a potential elicitor. For example, flagellin protein of *P. syringae* pv. *tomato* markedly induced HR cell death in tomato suspension culture (Taguchi *et al.*, 2003a). Genetic approaches revealed that flagellin is a major elicitor because a flagellin-defective mutant, *fliC* mutant of *P. syringae* pv. *tabaci*, was unable to induce a HR in tomato, and a HAP2-defective mutant, *fliD* mutant, of this pathogen secretes a large amount of monomer flagellins and induces a strong HR in tomato (Shimizu *et al.*, 2003; Taguchi *et al.*, 2003a, b). On the other hand, flagella were necessary for full virulence because the ability of the *fliC* mutant of *P. syringae* pv. *tabaci* to cause disease was significantly reduced in host tobacco leaves (Ichinose *et al.*, 2003a). Thus, flagellins are thought to be an important factor in determining compatible or incompatible relations between plants and *Pseudmonas syringae* (Ichinose *et al.*, 2003b). Also, Marutani *et al.* (2005) have been reported flagellin of *P. syringae* pv. *tabaci* is an elicitor that induces a hypersensitive reaction (HR) in nonhost tomato cells. Flagellin is the major HR elicitor produced by this pathogen, as shown by the inability of a flagellin-defective mutant, *fliC,* to induce HR. Also, a *fliD* mutant that secretes large amounts of monomer flagellins induces a strong HR in tomato. The possible involvement of an Hrp type III secretion system (T3SS) in flagellin-induced

HR was investigated using flagella-defective mutants or Hrp T3SS-defective mutants (Marutani *et al.*, 2005). The *hrcC* gene encodes HrcC protein, which is required for Hrp pilus formation in the outer membrane. An *hrcC* mutation, introduced into the wildtype, *fliC* and *fliD* mutants of *P. syringae* pv. *tabaci* did not affect swimming motility or flagellin secretion, whereas all *hrcC*, *fliC*, and *fliD* mutants lost the ability to cause disease on host tobacco leaves. However, the *hrcC* mutant and the *fliD* and *hrcC* double mutant were still able to induce HR cell death, expression of one of the defense-related genes *hsr203J*, and the generation of hydrogen peroxide in nonhost tomato cells. Thus, flagellin is required for both pathogenicity in host tobacco and HR in nonhost tomato. On the other hand, *hrp* T3SS is necessary for pathogenicity on host tobacco but is not indispensable to induce HR in nonhost tomato. These results clearly show that flagellin induced HR is *hrp*-independent in tomato (Marutani *et al.*, 2005).

As a result of host-pathogen coevolution, plants have developed sophisticated mechanisms to protect themselves from disease. Besides preformed physical and chemical barriers that hinder infection, a wide variety of defense responses is induced only after pathogen attack (Kombrink and Somssich, 1995). When these induced responses are triggered rapidly and coordinately during a given plant-pathogen interaction, the plant is resistant to disease. A susceptible plant responds more slowly with an onset of defense mechanisms after infection. Thus, the timely recognition of an invading microorganism and the rapid and effective induction of defense responses appear to make a key difference between resistant and susceptible plants (Yang *et al.*, 1997).

The activation of resistance in plants is initiated by host recognition of molecules called elicitors, which are directly or indirectly released from an invading pathogen. Although elicitors vary widely in their chemical composition (Ebel and Cosio, 1994), and the mechanisms by which plants perceive them may differ (Nürnberger *et al.*, 1994; Scofield *et al.*, 1996; Tang *et al.*, 1996). Many pathogen elicitors appear to trigger a common network of signaling pathways that coordinate the overall defense responses of plants. The induced mechanisms frequently manifest themselves as a hypersensitive response (HR), which is characterized by necrotic lesions resulting from localized host cell death at the site of infection. The HR prevents growth and the spread of the pathogen into healthy tissues (Goodman and Novacky, 1996).

The *xagrpoE* and *xagrpoN* affected extracellular enzyme activity was not different. They all reduced synthesis and secretion of alpha-amylase, cellulase, endoglucanase, pectate lyases, protease, and polygalacturonase activity. Previously reported the σ^E or sigma^E of *E. coli* and several bacteria are generally through to be a global regulator and have been demonstrated to be involved in the adaptation to environmental stresses and regulation of the expression, function or secretion of extracellular enzymes and virulent factors (Helmann, 2002). It was of interest to investigate the role of σ^E played in *X. axonopodis* pv. *glycines* 12-2. We found that the σ^E mutant had affected to the extracellular enzyme activities (data not shown). Bacterial sigma (σ) factors are an essential component of RNA polymerase and determine promoter selectivity. The substitution of one sigma factor for another can redirect some or all of the RNA polymerase in a cell to activate the transcription of genes that would otherwise be silent. As a class, alternative sigma factors play key roles in coordinating gene transcription during various stress responses and during morphological development (Helmann, 2002). It was of interest to investigate the role of σ^E played in *X. axonopodis* pv. *glycines* 12-2. We found that the σ^E mutant had affected to the extracellular enzyme activities (data not shown). Bacterial sigma (σ) factors are an essential component of RNA polymerase and determine promoter selectivity. The substitution of one sigma factor for another can redirect some or all of the RNA polymerase in a cell to activate the transcription of genes that would otherwise be silent. As a class, alternative sigma factors play key roles in coordinating gene transcription during various stress responses and during morphological development (Helmann, 2002).

E. carotovora subsp. *carotovora* strain 71, HrpL$_{Ecc}$, an alternate sigma factor of the extracytoplasmic function subfamily, plays a central role in the expression of the *hrp* regulon that virulence factors like extracellular enzymes are included. Chatterjee *et al.* (2002) reported the sigma-54 (RpoN) is required for full expression of *hrpL*$_{Ecc}$ and that HrpS, in conjunction with sigma-54, activates *hrpL*$_{Ecc}$

transcription. They also made the novel observation that integration host factor is required for the activation of the *hrpL*$_{Ecc}$ promoter and findings reveal that the RsmA/*rsmB* RNA-mediated post-transcriptional system, known to control extracellular enzyme and harpin production, affects *hrpL*$_{Ecc}$ expression as well. Based upon the data now available for *E. carotovora* subsp. *carotovora* and extrapolating from the evidence in other systems, we propose a tentative model that depicts the Hrp regulatory system of *E. carotovora* subsp. *carotovora* and explains the basis for coregulation of extracellular enzyme production and expression of the Hrp regulon (Chatterjee *et al.*, 2002).

The bacterial population of both *xagrpoE* and *xagrpoN* was significantly lower than the wildtype at 3, 4, and 5 day after inoculation. The *xagrpoN* mutant appeared to be rescued its growth. The results indicate that direct or indirect gene expression for epiphytic fitness of *X. axonopodis* pv. *glycines* may be under more control by *xagrpoE* than *xagrpoN*. Kenyon *et al.* (2002) indicated that C-starvation appears to generate signals capable of inducing the σ^E regulon and that one or more members of the σ^E regulon are required for the two known functions of the SSR, LT-CSS and CSI cross-resistance to other environmental stresses in *Salmonella enterica* serovar Typhimurium. A previous report (Humphreys *et al.*, 1999) suggested that *rpoE* mutants might be defective in the utilization of certain C-sources. This was inferred from the findings that an *rpoE* mutant, compared to wildtype strains, exhibited a longer lag phase and failed to reach as high an optical density after overnight growth in LB or M9 succinate medium in contrast to LB glucose or M9 glucose medium, respectively. Kenyon *et al.* (2002) suggests that *rpoE* mutants might be deficient in the use of oxidatively metabolizable C-sources. Moreover the σ^E controls cell lysis and the amount of nutrients released from dead cells to maintain the viability of a subpopulation of viable cells.

The *rpoN* mutant was found to be nonmotile, suggesting a role of RpoN in *A. brasilense* flagellar biosynthesis (Milcamps *et al.*, 1996). The *rpoN* mutant in *X. axonopodis* pv. *glycines* 12-2 however, was not completely lost motility. Ralston (2008) reported the environmental influences on gene expression. Bacterial genes are organized into operons, or clusters of coregulated genes. The formation of pili and flagella in *P. aeruginosa* is under the control of RpoN. All *rpoN* mutants showed significant reduction of adherence to epithelial cells and tracheobronchial mucin (Chi *et al.*, 1991; Ramphal *et al.*, 1991; Simpson *et al.*, 1992). RpoN mutants have been used as controls defective in adherence in many adhesion experiments and infection models (Comolli *et al.*, 1999). Moreover, RpoN negatively regulates the expression of *sadB*, an essential gene for surface adhesion (Caiazza and O'Toole, 2004). The interaction of RpoN with PilR, a member of a two-component transcriptional regulatory system, was shown to control the expression of type 4 fimbriae (Mattick *et al.*, 1996). The presence of *pilRS* and *rpoN* genes is required for the expression of *pilA*, the structural gene for type IV pilin, whereas any of these are essential for *pilBCD* expression (Boyd and Lory, 1996; Koga *et al.*, 1993). Flagella, as well as type IV pili, are also important structures involved in attachment and RpoN was shown to control flagella-based adhesion and motility (Totten *et al.*, 1990). Studies indicate that both motility and adhesion are regulated by a two-component regulatory system called *fleRS*, which in turn is controlled by another regulator in a cascade that involves *rpoN*. RpoN coupled with the action of the transcriptional activator FleQ is involved in the expression of *fliD*, the flagellar cap protein involved in mucin adhesion (Arora *et al.*, 1998; Jyot *et al.*, 2002).

Microorganisms are able to survive under a wide range of environmental conditions (e.g., osmolarity, temperature, and nutrient availability) by rapidly adapting their structure and physiology (Soutourina *et al.*, 2001). These mechanisms are based on the existence of multiple regulatory systems in which gene expression is controlled in a coordinate manner in response to environmental stimuli. One example of such a complex process is the regulation of motility and chemotaxis in bacteria (Macnab, 1996). More than 80% of the known bacterial species are motile by means of flagella (Moens *et al.*, 1996). The structure and arrangement of flagella differ from species to species and seem to be related to the specific environments in which the cells live (Wilson and Beveridge, 1993). Flagella can be arranged on the cell body in a variety of configurations, including single polar, multiple polar, and many peritrichous (or lateral) configurations. Motility by means of flagella is thought to provide a specific advantage for a bacterium (Moens *et al.*, 1996), because it helps the bacterium to reach the most favorable environment and to successfully compete with other microorganisms. However, the cost of maintenance of a flagellar motility system is high for bacteria (about 2% of biosynthetic energy expenditure in *E. coli*) due to the number of genes and the energy required for flagellum synthesis and functioning. As a result, the flagellar system is highly regulated (Macnab, 1996). Given its importance for bacterial survival under specific conditions, the efficiency of control of this complex system seems to be under strong selective pressure in the environment. In addition to being physically close in the genome, these genes are regulated such that they are all turned on or off together. Grouping related genes under a common control mechanism allows bacteria to rapidly adapt to changes in the environment (Ralston, 2008).

The *xagrpoE* and *xagrpoN* mutant showed reduced disease severity was measured at 10 days after inoculation on soybean cv. Spencer with 11.1 and 14.8% disease severity respectively, compared to wildtype. Hendrickson *et al.* (2001) by modulating different virulence determinants, RpoN has been strongly linked to the virulence of *Pseudomonas aeruginosa* as well as to its ability to efficiently colonize several hosts including mammals, insects, nematodes and plants (Hendrickson *et al.*, 2001). All these RpoN-regulated genes have a consensus promoter sequence of -24(GG)/-12(GC); their expression required at least one transcriptional activator (Thony and Hennecke, 1989). Hence, the sigma-54 like alternative sigma factor RpoN is implicated at different stages for the regulation of *P. aeruginosa* virulence factors. Mutations in *rpoN* in all cases lead to a reduction in virulence; this is visualized as defects in adhesion, motility and cell-to-cell communication observed in several models of infection (Hendrickson *et al.*, 2001).

In addition a subgroup of sigma factors in the extracytoplasmic function (ECF) family regulates the expression of genes associated with the ability of the microorganism to respond to environmental changes, and in some cases these genes are also involved in bacterial virulence (Kazmierczak *et al*., 2005). The presence of 13 genes coding for sigma factors in *Mycobacterium tuberculosis* is an indication of the ability of this microorganism to adapt to different stress conditions. The expression of these genes has been characterized (Manganelli *et al.*, 1993), and the role played by many of these sigma factors in *M. tuberculosis* virulence has been extensively analyzed (Rodrigue *et al.*, 2006). Interestingly, although mutants have been created for different ECF sigma factors of *M. tuberculosis*, only the σ^E mutant is attenuated in resting and activated macrophages, suggesting that this regulator controls genes directly related to the intracellular survival of the bacterium. In many bacterial species, sigma factors similar to σ^E have been identified as master regulators responding to extracellular stress and controlling the expression of genes relevant for pathogenesis (Rowley *et al.*, 2004). Expression of σ^E in *M. tuberculosis* is up-regulated under SDS, oxidative, and heat shock stress, and an *M. tuberculosis sigE* mutant is more sensitive to these conditions (Manganelli *et al*., 1993).

A *xagP* has been interfered with alpha-amylase, cellulase, protease gene expression, and correlated with the amount of enzyme activities decreased in *xagP* mutant**.** *FliC* also be related to the release or secrete of the enzyme is effective in inhibiting the production of bacterial enzymes *X. axonopodis* pv. *glycines* 12-2 by inhibited enzyme genes expression, especially cellulase. This information to confirm the *xagP* gene not only affected to pectase lyase production but also affected on other key enzymes involved virulence and pathogenicity factors of *X. axonopodis* pv. *glycines* 12-2 including; alpha-amylase, cellulase, and protease. Casadevall and Pirofski (2009) reported that the numerous enzymes have been implicated in microbial virulence. Enzyme that are considered virulence factors are generally active against host components and contribute to virulence by damaging host tissue. Tissue damage makes the host permissive for microbial infection. Enzyme virulence factors that damage tissue such as protease. This enzyme damage cells and provide nutrients by digesting substrates into smaller components that can be assimilated by microbes (Casadevall and Pirofski, 2009). However, Cox *et al.* (2000) other enzymes, such as urease, contribute to virulence by facilitating survival inside phagocytic cells. Previously reported the σ^E or sigma^E of *E. coli* and several bacteria are generally through to be a global regulator and have been demonstrated to be involved in the adaptation to environmental stresses and regulation of the expression, function or secretion of extracellular enzymes and virulent factors (Ching *et al.*, 2008; Helmann, 2002). It was of interest to investigate the role of σ^E played in *X. axonopodis* pv. *glycines* 12-2. The σ^E mutant had affected to the extracellular enzyme activities (data not shown). Bacterial sigma (σ) factors are an essential component of RNA polymerase and determine promoter selectivity. The substitution of one sigma factor for another can redirect some or all of the RNA polymerase in a cell to activate the transcription of genes that would otherwise be silent. As a class, alternative sigma factors play key roles in coordinating gene transcription during various stress responses and during morphological development (Helmann, 2002).

The results showed down-expression of *flgC*, *fliC*, and *xagP* in *xagrpoE* and xag*rpoN* except *fliD* gene in *xagrpoE* mutant that showed similar expression with wildtype. This result correlated with *xagrpoE* and *xagrpoN* that have defected extracellular enzymes secretion especially pectate lyase. They were influent on virulence with 11.1 and 14.8% increased disease severity on Spencer compared to wildtype. Mark *et al.* (2005) report the sigma factors provide promoter recognition specificity to RNA polymerase holoenzyme, contribute to DNA strand separation, and then dissociate from the core enzyme following transcription initiation. As the regulon of a single sigma factor can be composed of hundreds of genes, sigma factors can provide effective mechanisms for simultaneously regulating expression of large numbers of prokaryotic genes. One newly emerging field is identification of the specific roles of alternative sigma factors in regulating expression of virulence genes and virulence-associated genes in bacterial pathogens (Mark *et al.*, 2005). Virulence genes encode proteins whose functions are essential for the bacterium to effectively establish an infection in a host organism. In contrast, virulence-associated genes can contribute to bacterial survival in the environment and therefore may enhance the capacity of the bacterium to spread to new individuals or to survive passage through a host organism. As alternative sigma factors have been shown to regulate expression of both virulence and virulence-associated genes, these proteins can contribute both directly and indirectly to bacterial virulence. Sigma factors are classified into two structurally unrelated families, the σ^{70} and the σ^{54} families. The σ^{70} family includes primary sigma factors (e.g., *Bacillus subtilis* σ^A) as well as related alternative sigma factors; σ^{54} forms a distinct subfamily of sigma factors referred to as σ^{N} in almost all species for which these proteins have been characterized to date. We present several examples of alternative sigma factors that have been shown to contribute to virulence in at least one organism. For each sigma factor, when applicable, examples are drawn from multiple species (Mark *et al.*, 2005).

CONCLUSION

This work leads to the fact of *X. axonopodis* pv. *glycines* 12-2 pathogenicity resulting development of control measure on soybean bacterial pustule as the following details:

1. A genetic and functional characterization of the flagellin genes including flagella hock (*flgC*), flagellin (*fliC*), and hock associated protein 2 (*fliD*) and pectate lyase (*xagP*) genes of *X. axonopodis* pv. *glycines* strain 12-2 were clearly identified. Mutations in *flgC, fliC, fliC&fliD,* and *fliD&fliC* resulted in loss of swimming motility, whereas *fliD* and double mutant *fliD&xagP* showed reduced motility.

2. The *xagP* mutant did not differ in motility from *X. axonopodis* pv. *glycines* wildtype but produced a reduced bioflim. The *xagP* and double mutant *fliD&xagP* mutants completely lost ability to produce pectolytic activity, where *flgC, fliC, fliC* and *fliD,* and *fliD&fliC* revealed reduced pectolytic activity on potato slices.

3. The *flgC, fliC, fliC&fliD,* and *fliD&fliC* mutants did not express flagellin visualized as a 34.5-kDa band on SDS-PAGE gels. They were however, essential for both swimming motility and translocation of virulence factors that referred as T3SS. The *flgC* might function as flagellin secretion switch off, where *fliC* and *fliD* genes function as injectisome delivering protein effectors into the plant. It is possible that the pump complex of virulence factors and the export machinery form independently of each other. In this study, *fliC* and *fliD* cannot be function without the pump complex and this is difficult to explain why *flgC* mutant is nonflagellate, remaining to challenged investigation.

4. The *flgC, fliC* and *fliD* mutants did not affect development of a hypersensitive response (HR) in tobacco leaves (*Nicotiana glauca*, *N. rustica*, and *N. tabacum* cv. Xanthi), chili, and tomato plant unlike *xagP* and double mutant *fliD&xagP* mutants could not induced HR on tobacco plant.

5. All *flgC, fliC, fliD, xagP, fliC&fliD, fliD&fliC*; and *fliD&xagP* mutants obtained from *X. axonopodis* pv. *glycines* 12-2 wildtype altered the production of extracellular enzymes including cellulase, alpha-amylase, endoglucanase, and protease. Mutants *flgC, fliC&fliD,* and *fliD&fliC* completely lost pathogenicity, and reduced biofilm formation, growth in soybean, and disease severity on leaves than single mutant, including caused HR on soybean cv. Spencer. The single mutants, *flgC, fliC, fliD*, *xagP*, and double mutations (*fliC&fliD, fliD&fliC, fliD&xagP*) were exhibited significantly reduced biofilms on similar surfaces and confirmed for bioflim formation on glass slide under compound microscope. Disease severity caused by *fliC, fliD*, *xagP*, and *fliD&xagP* mutants was reduced with 96.8, 72.6, 95.6, and 98.1% respectively. Complementation of all mutants restored full virulence. Complementation of *flgC, fliC, fliD, fliC&fliD, fliD&fliC,* and *xagP&fliD* restored motility and complementation of *xagP* and double mutant *fliD&xagP* restored HR.

6. The nonmotile single mutant *flgC*, *fliC* and double mutants including *fliC&fliD, fliD&fliC*, and *fliD&xagP* were reduced disease severity found on Spencer cultivar seem like they were lost pathogenesis and were nonpathogenic strains. The population of *flgC, fliC, fliC&fliD, fliD&fliC* and *fliD&xagP* mutants on soybean cv. Spencer were significantly decreased, reaching population numbers lower than wildtype. The mutant *fliD* and all of complemented strains were not different when observed on soybean cv. Spencer leaves daily up to 7 days after infiltration.

7. Analysis of enzymatic gene expression (alpha-amylase, cellulase, and protease) in *xagP* and *fliC* mutant strains, was found that the *xagP*; and *fliC* have affaced to suppress expression of alpha-amylase, cellulase, and protease genes with 11.6, 16.6, and 19.9; and 3.6, 25.8, and 10.1 % respectively. The *xagP* has been interfered with alpha-amylase, cellulase, protease gene expression, and correlated with the amount of enzyme activities decreased in *xagP* mutant. *FliC* also related to the released or secreted enzymes that was effective in inhibiting gene expression of enzyme activity, especially cellulase. In all cases, *fliC* showed strongly decrease in cellulase activity, where *xagP* significantly (P=0.05, DMRT) repressed the expression of alpha-amylase and protease genes. From this investigation, it is possible that *xagP*

exhibited a greater impact than *fliC* on the activity of these 3-detected enzymes examined. These results confirmed that *xagP* and *fliC* genes not only affected pectase lyase and flagellin production but also affected on other key enzymes involved in virulence and pathogenicity factors of *X. axonopodis* pv. *glycines* 12-2 including; alpha-amylase, cellulase, and protease. Threrfore, the genes respond to these extracellular enzymes are related to *xagP* and *fliC*. These innovative findings shown by RT-PCR analysis demonstrate that *xagP* and *fliC* have specific activity to repress gene expression of the 3-target enzyme activities resulting co-regulated reduction of virulence and pathogenicity in *X. axonopodis* pv. *glycines* 12-2.

8. Global genetic basis among genes *XagP*, *fliC*, *fliD*, *flgC*, extracelluar enzymes (alpha-amylase, cellulase, and protease) and *rpoE* and *rpoN* in *X. axonopodis* pv. *glycines* 12-2 were characterized for the first time. RpoE; and RpoN ruled the expression of XagP; and FliC respectively that were necessary for transcriptional regulators for activating multiple virulence genes and virulence-associated genes. Both *rpoE* and *rpoN* therefore, played a role in *X. axonopodis* pv. *glycines* 12-2 infection of soybean bacterial pustule disease. However, it remains to be investigated in future studies if the *X. axonopodis* pv. *glycines* 12-2 genome sequence revealed more clues for the mechanism by which flagella genes affect *xagP* including different extracellular enzyme activities and production. Additional experiments of these associated genes (e.g. genes involved in extracellular enzymes) should conduct artificial gene silencing. The present understanding is however, insufficient to fully demonstrate the roles of affected enzyme and flagella protein expression.

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APPENDIX

Recipes of media, antibiotic, and buffer in this study

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 or nutrient broth may be purchased in dehydrated form Difco.

* Do not add agar if nutrient broth is desired.

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.

3. 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.

4. SOB medium

	per L
Bacto-tryptone	20.0 g
Bacto-yeast extract	5.0 g
NaCl	0.5 g
1M KCl	2.5 mL
ddH$_2$O	1000 mL

*****Note: adjust pH to 7.0 with 10N NaOH, autoclave to sterilize, and add 10 ml of 1 M MgCl$_2$ before use

5. SOC medium

	per L
Bacto-tryptone	20 g
Bacto-yeast extract	5 g
NaCl	0.5 g
1M KCl	2.5 mL
ddH$_2$O	1000 mL

*Note: adjust pH to 7.0 with 10N NaOH, autoclave to sterilize, add 20 mL of sterile 1 M glucose immediately before use

Antibiotic

1. **Kanamycin (25mg/ml)**

Kanamycin sulfate	0.25 g
Sterile ddH$_2$O	10 mL
Total volume	10 mL

* Note: aliquot and store at -20 degree

2. Ampicillin (50 mg/ml)

Ampicillin	0.5 g
Sterile ddH$_2$O	10 mL
Total volume	10 mL

*Note: aliquot and store at -20 degree

3. Gentamycin (10 mg/ml)

Gentamycin	100 mg
ddH$_2$O	10 mL
Total volume	10 mL

*Note: Filter sterilize, alliquot and store at -20 degree

Buffer

1. HEPES buffer

	Per L
1M HEPES-KOH	60.0 mL
500 nM Tris-HCl	40.0 mL
0.5 M EDTA	10.0 mL
DMSF	0.435 g
DTT (Dithiothretol)	0.386 g
Glycerol (100%)	500.0 mL
2M KCl	125.0 mL

*Adjust pH to 7.5 with potassium hydroxide (KOH) and store at 4 degree

2. TBE (10X)

Tris base	216 g
Boric Acid	110 g
EDTA	16.6 g
Water to	2.0 L
Total volume	2.0 L

3. Coomassie Stain

Coomassie R250	1g
glacial acetic acid	100 mL
methanol	400 mL
ddH_2O	500 mL

4. De-Stain for Coomassie

Methanol	200 mL
glacial acetic acid	100 mL
ddH_2O	700 mL

5. Acrylamide range 12%

22.2% Acrylamide/0.6% Bis	10.81 mL
1M Tris/HCl pH=8.8	7.5 mL
Distilled water	1.38 mL
10% SDS	200 µL
10% Ammonium Persulfate	100 µl
TEMED	10 µl

6. 22.2 % Acrylamide/Bisacrylamide mix

Acrylamide	22.2 g
Bis-acrylamide	0.6 g
ddH$_2$O	100 mL

7. 44.4 % Acrylamide/Bisacrylamide mix

Acrylamide	44.4 g
Bis-acrylamide	1.2 g
ddH$_2$O	100 mL

8. Reservoir/Running buffer

Glycine	57.6 g
Tris base	12 g
SDS	4 g
ddH$_2$O	4 L

9. Stain solution

Coomassie Brilliant Blue R-250	2.5 g
Methanol	450 mL
Glacial acetic acid	100 mL
ddH$_2$O	1 L

10. Destain solution

Methanol	300 mL
Acetic acid	400 mL
ddH$_2$O	4 L

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