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Vol. 7(43), pp. 4990-5003, 25 October, 2013
DOI: 10.5897/AJMR2013.5671
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Hemin transported protein of *Xanthomonas axonopodis* pv. *glycines* functions on leaf colonization and virulence on soybean

Sutruedee Prathuangwong[1]*, Dusit Athinuwat[2], Wilawan Chuaboon[1], Lawan Kladsuwan[1], Malida Choorin[1] and Tiyakhon Chatnaparat[1]

[1]Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.
[2]Major of Organic Farming Management, Faculty of Science and Technology, Thammasat University, Pathumthani 12121, Thailand.

Accepted 20 September, 2013

***Xanthomonas axonopodis* pv. *glycines* (Xag) causes bacterial pustule disease on soybean. This bacterium is present worldwide around hot and humid growing regions such as Southeast Asia. To understand if the gene coding for hemin transport protein (*hem*) is involved in virulence of the pathogen in soybean, we generated a *hem* mutant in Xag by overlapping PCR mutagenesis. Disruption of *hem* significantly reduced the population size and the disease incidence when sprayed on soybean but not when injected directly to soybean. The *hem* mutant caused the hypersensitive response induction on tobacco as an Xag wildtype. Interestingly, the *hem* expression was also reduced when the Xag wildtype grow *in planta*. The hemin transporter protein involved in the production of extracellular polysaccharide, biofilm formation, motility and attachment but not for extracellular enzymes. This confirmed that epiphytic fitness of Xag strongly required *hem* functions. These results suggest that *hem* gene is essential for virulence of Xag on soybean during the infection process.**

Key words: Bacterial pustule disease, virulence factors, iron uptake system, *hem* gene, epiphytic fitness.

INTRODUCTION

Bacterial diseases of soybean appear worldwide and cause production losses and decreases yield by reducing quality and quantity. The most common bacterial disease of soybean is bacterial pustule, caused by *Xanthomonas axonopodis* pv. *glycines*, is one of the most serious diseases of soybean in several part of soybean production areas including Thailand. Bacterial pustule lesions are small pale green spots with raised centers on either or both leaf surfaces. The bacterial pustule lesions may enlarge and coalesce, leading to premature defoliation (Narvel et al., 2001). Severe disease causes yield losses up to 40% (Prathuangwong and Amnuaykit, 1989). *X. axonopodis* pv. *glycines* infects the soybean plant through stomata and wounds. After invasion into the plant, bacteria multiply within intercellular spaces of the spongy mesophyll for pustule induction on susceptible soybeans (Jones and Fett, 1985).

Nutritional conditions are reported to be an important virulence factors for the disease induction of plant pathogenic bacteria. For example, the translations of pathogenicity island (*hrp* gene cluster) of *Xanthomonas* are induced by sucrose and sulfur-containing amino

*Corresponding author. E-mail: agrsdp@ku.ac.th.

acids (Schulte and Bonas, 1992). While iron is an essential element for pathogenic bacteria due to its participation in the tricarboxylic acid cycle, electron transport, amino acid and pyrimidine biosynthesis, DNA synthesis, and other critical functions (Lemanceau et al., 2009). Moreover, iron is considered to play a critical role in plant-bacterial interactions. During bacterial infection, there is aggressive competition between the plant and the bacteria iron can play a critical role in such competitive relationships (Lemanceau et al., 2009). Segond and collaborators found that metal transporter AtNRAMP3 in Arabidopsis is upregulated in leaves challenged with the *Pseudomonas syringae* and *Erwinia chrysanthemi* (Segond et al., 2009). Similarly, *A. thaliana* synthesizes the ferritin AtFER1, an iron storage protein, is required for Arabidopsis resistance to *E. chrysanthemi* infection (Boughammoura et al., 2007).

Iron is one of the factors that limits bacterial growth *in planta* because concentrations of iron are necessary to support bacterial growth and multiplication (Expert et al*.,* 1996). Siderophore-mediated transport of iron is one of the mechanisms used by bacteria to uptake iron from their environment (Braun et al., 1998; Lee, 1995; Mietzner and Morse, 1994). Thus, the production of a siderophore by bacterial pathogens could significantly deplete the iron reserves of the host plant and weaken host-defence reactions (Lemanceau et al., 2009). Several studies have shown the importance role of iron in virulence of plant pathogenic bacteria. For instance, siderophore-deficient mutants of *Erwinia amylovora*, *E. chrysanthemi* strain 3937, *Erwinia carotovora* subsp. *carotovora*, *Ralstonia solanacearum* and *Agrobacterium tumefaciens* are virulence deficient on its host plants (Dellagi et al., 1998; Franza et al., 2005; Bhatt and Denny, 2004; Bull et al., 1996; Rondon et al., 2004). However, *P. syringae* pv. *syringae* B301D and *P*. *syringae* pv. *tomato* DC3000 do not show any growth defect or alter virulence on host plant (Jones et al., 2007; Jones and Wildermuth; 2011). In addition to the uptake of iron, several *Xanthomonas* take up iron via *tonB* system. In *Xanthomonas campestris* pv. *campestris*, mutation of *tonB*, *exbB* and *exbD1* genes which are involved in iron uptake system have been reported to be impaired for ferric ion uptake and exhibited reduced virulence in cabbage (Wiggerich and Puhler, 2000). The *fur* mutant of *Xanthomonas oryzae* pv. *oryzae* is virulence deficient and hypersensitive to oxidative stress (Subramoni and Sonti, 2005). Different pathways of iron uptake from direct Fe^{2+} transport and host iron binding proteins or heme also may be employed by pathogenic bacteria (Ratledge and Dover, 2000; Velayudhan et al., 2000).

Hemin is one of heme oxidized form that consists of an iron ion and found in extracellular environments (Lee, 1995). Hemin iron transport and utilization systems have been identified in numerous bacterial species, where it was shown that an outer membrane receptor and a periplasmic binding protein-dependent ABC-type transporter are required for hemin uptake (Stojiljkovic and Hantke, 1992, 1994). It is the cofactor in reactions involved in various cellular functions including oxygen transport and electron transfer (Lee, 1995).

For phytopathogenic bacteria, *Xylella fastidiosa* 9a5c contains 67 genes encoding proteins involved iron metabolism and has been reported to contain five membrane receptors, including siderophore, ferrichrome-iron and hemin receptors, all of which are thought to be associated with iron transport, utilization and virulence (Simpson et al., 2000). Whereas, the extensive genetic and genomic resources are available for *X. axonopodis* pv. *glycines*, and it has been the subject of highly productive research centered on the mechanisms of plant host susceptibility/resistance and pathogen virulence and avirulence determinants (Athinuwat et al., 2009; Chatnaparat et al., 2012; Kasem et al., 2007; Kaewnum et al., 2005; 2006; Thowthampitak et al., 2008). Recently, the draft genome of *X. axonopodis* pv. *glycines* 12-2 has been sequenced and found that this strain contains genes encoding hemin uptake locus. Although, the effects of iron uptake system in cell growth and virulence production of several bacteria plant pathogens have been documented, hemin transport protein (*hem*) of the bacteria that involved in the infection process of plant are not established. Thus, in this study, a *hem* mutant was constructed using overlapping extension mutagenesis. The roles of *hem* in contribute to full virulence of Xag on soybean were investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids and recombinant techniques

Bacterial strains and plasmids used in this study are described in Table 1. *X. axonopodis* pv. *glycines* wildtype strain 12-2 was cultured at 28°C in nutrient glucose agar (NGA) (Sambrook et al., 1989). Mutants were cultured on NGA containing 50 μg/ml kanamycin and 50 μg/ml chephalexin. The complemented *hem* was cultured on NGA containing 50 μg/ml kanamycin, 50 μg/ml cephalexin, and 40 μg/ml gentamycin. All DNA manipulations including DNA isolation, plasmid extraction, restriction digestion, ligation, and gel electrophoresis were performed as described previously (Sambrook et al., 1989).

Knockout of *hem* genes in *X. axonopodis* pv. *glycines* 12-2

A disruption of gene coding for hemin transport protein (*hem*) was accomplished using overlap extension mutagenesis (Figure 1). The upstream and downstream regions of *hem* gene in *X. axonopodis* pv. *glycines* 12-2 were amplified using HemKO-1-F and HemKO-1-R primers that unique to upstream region and HemKO-2-F and HemKO-2-R primers that unique to downstream region of *hem* gene respectively (Table 2), with one having an extension complementary to the kanamycin resistance cassette from pKD13 to generate two amplicons with ends overlapping those of the resistance cassette (Datsenko and Wanner, 2000). Overlap

Table 1. Bacterial strains and plasmids.

Bacterial strain or plasmid	Relevant characteristic	Reference or source
X. axonopodis* pv. *glycines		
12-2	Wildtype, soybean pathogen	Thowthampitak et al. (2008)
hem mutant	Km^r, *hem*-::Kan, 12-2 derivative	This study
hem+	*Hem* mutant complemented with pBBR::*hem*	This study
Plasmids		
pTok2	ColE1 replicon, suicide plasmid, Tc^r	Kitten and Willis, 1996
pKD13	FRT-Km^r-FRT, oriR6K, Ap^r, Km^r	Datsenko and Wanner (2000)
pBBR1MCS-5	Broad host range cloning vector, *lacZ*, Gm^r	Kovach et al. (1995)
pTok2::Δ *hem*	Δ *hem*::Kan from overlapping PCR cloned into pTok2, Tc^r, Km^r	This study

extension PCR was used to link the two PCR amplicons and the resistance cassette; this larger fragment was then cloned into the destination vector pTok2 using the quick ligation protocol (New England Biolabs Inc.) and introduced into *E. coli* S17-1, the mobilizing strain by transformation, then transferred to *X. axonopodis* pv. *glycines* via conjugation, selecting for transconjugants on NGA containing 50 μg/ml kanamycin and 50 μg/ml chephalexin as a sensitive antibiotic for *E. coli* strain. Gene disruption was confirmed using PCR, with primers specific (Hem-C-F and Hem-C-R) to the sequences flanking of *hem* gene (Table 2).

Complementation of *hem* mutants

To complement the *hem* mutant, 800 bp of *hem* containing the native promoter was amplified using primers *hem* com-F and *hem* com-R (Table 2). The amplicon was digested with *Hin*dIII and ligated into the multiple cloning site of vector pBBR1MCS-5 to yield pBBR::*hem,* which was then introduced into *hem* mutant by electroporation. The complemented *hem* mutant was cultured on NGA containing 50 μg/ml kanamycin, 50 μg/ml cephalexin, and 40 μg/ml gentamycin and also confirmed by PCR using primers *hem* com-F and *hem* com-R.

Real-time quantitative reverse transcription PCR analysis

The expression of *hem* was determined by real-time qRT-PCR of cDNA isolated from both an Xag wildtype and a *hem* mutant grown in nitrogen yeast glycerol broth (NYGB) for 24 h as well as in *X. axonopodis* pv. *glycines* cells recovered from infected soybean plants. For *in planta* experiment, Xag cells in soybean were isolated from the leaves according to the method described by Yu et al. (2013). Briefly, the bacterial cells at 1×10^8 cfu/mL were introduced by vacuum infiltration into soybean leaves. Infiltrated plants were incubated for 4 days. A total of 150 to 200 leaves were collected cut into squares, and submerged in an acidic phenol RNA-stabilizing solution. The solution was filtered and centrifuged to harvest the bacterial pellets. Total RNA preparation for using in the real-time qRT-PCR was isolated with TRIzol (Invitrogen Life Technologies) from cells grown in NYGB and in *planta* using the method of Santiago-Vazquez and associates (2006). cDNA was generated from 1 μg of RNA using SuperScript II (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) and random hexamers. Real-time qRT-PCR was performed on 1 μg of the cDNA using LightCycler FastStart DNA Master PLUS SYBR Green I (Roche, Indianapolis, IN, U.S.A.) on a Roche Lightcycler II (Roche) following the manufacturer's specifications. The specific primer pairs in this experiment are list in the Table 2. An external standard curve was generated using purified *ihfA* (integration host factor A) DNA (Champoiseau et al., 2006). Melting curve analysis was used to verify amplification of a single product. The concentration of amplification products from negative controls (RNA samples to which no superscript was added) was undetectable in all cases, indicating a lack of interference from contaminating DNA.

High iron concentration sensitivity assay

To test for high iron concentration sensitivity, an Xag wildtype, a *hem* mutant and a complemented *hem* mutant were grown in NYGB at 28°C with shaking at 200 rpm to an optical density (OD) at 600 nm of 1.0 (OD_{600} = 1.0). Cultures were transferred to NYGB supplemented with $FeCl_3$ to a series of final concentrations at 0, 4, 5, and 6 mM, respectively and were incubated at 28°C with shaking at 200 rpm. After incubation for 24 h, the cell density was measured spectrometrically using a spectrophotometer and absorbance at OD_{600} was determined (Yang et al., 2007). The experiments were repeated three times with at least three replicates in each experiment.

The extracellular polysaccharide (EPS) production

EPS production of an Xag wildtype, a *hem* mutant and a complemented *hem* mutant was measured with some modification as described by Tang et al. (1991). Cultures were grown in NYGB containing 4% glucose at 28°C with shaking at 200 rpm for 5 days. EPS was precipitated from the culture supernatant with ethanol. Then EPS was dried at 80°C to constant weight and the difference between the two weights was used to estimate the production of EPS per millilitre culture. The experiments were repeated three times with at least three replicates in each experiment.

Biofilm formation

Cells of *X. axonopodis pv. glycines* strains taken from cultures grown on nitrogen yeast glycerol agar (NYGA) for 24 h were suspended in NYGB and cell suspension of each strain was added

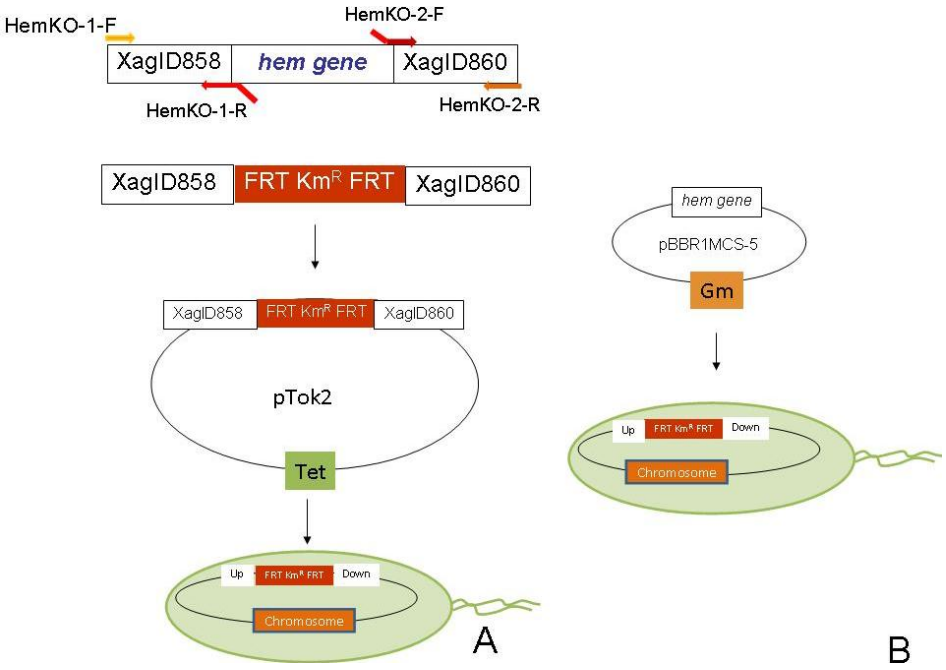

Figure 1. Overlap extension PCR was used to create constructs in the suicide-delivery vector pTOK2 to create site-directed mutant of *hem* gene in *Xanthomonas axonopodis* pv. *glycines* 12-2 by recombination (A). A *hem*-complementary strain**,** the 0.8-kb sequence of *hem* containing the native promoter was amplified and ligated into the multiple cloning site of vector pBBR1MCS-5(B).

Table 2. Polymerase chain reaction primers.

Primer	Sequence[a]	Description
HemKO-1-F	5' CGAAGACTATGGCAGCATC 3'	Amplification of upstream region of *hem* for generate *hem* mutant
HemKO-1-R	**5' GAAGCAGCTCCAGCCTACACA** GGTGTAATAGCTGTCCAGCTG 3'	……..
HemKO-2-F	5' GGTCGACGGATCCCCGGAAT GTTGTATCGCACTGACGCTG 3'	Amplification of downstream region of *hem* for generate *hem* mutant
HemKO-2-R	5' CTTCGAATACTGCCTGCAG 3'	……
hem com-F (*Hin*dIII)	5' TTGTAGTAAAGCTT ATTCGCGCCTGGCGCCAAG 3'	Amplification of 0.8 kb of *hem* for generate *hem* complementary strain
hem com-R (*Hin*dIII)	5' TTGTAGTAAAGCTT AGCGTCAGTGCGATACAACG 3'	……
Hem-C-F	5'CTGGCGGTGAGCTGGTAG 3'	Primers specific to the sequences flanking of *hem* gene
Hem-C-R	5'AGCGTCAGTGCGATACAAC 3'	……
*hem*RT-F	5'ACGAACTGGATGGCTGGCAGC 3'	Specific primer for *hem* expression using RT-PCR
*hem*RT-R	5' CGGCCGTCGATGCCCTCTTC 3'	……
*hrpF*RT-F	5' GACTCCATTTCCAAGGACGA 3'	Specific primer for *hrpF* expression using RT-PCR
*hrpF*RT-R	5' GCGCTCATGTTGTCGTAGAA 3'	……
*hrpD*RT-F	5' CGGTCACCCAAGATATGAGC 3'	Specific primer for *hrpD* expression using RT-PCR
*hrpD*RT-R	5' CATTGAAGTCGTTGCGTGAG 3'	……

[a]Bold sequence= Reverse complementary priming for pKD13 site, underlined sequence= pKD13 priming site.

to glass tubes, and grown at 28°C for three days. The presence of a biofilm was visualized as a white ring on the tube side wall, usually at the air-medium interface and quantified by crystal violet staining as previously described (Davey and O'Toole, 2000). Dye abundance was measured by absorption at 570 nm using a spectrophotometer. Readings from five replicates were averaged. The experiments were repeated three times with similar results.

Bacterial attachment

Bacterial adhesion of *X. axonopodis* pv. *glycines* strains to soybean leaves was assessed by immersing six individual leaves into 500 ml of a suspension of a given bacterial strain (10^7 cells/ml) at 28°C. After 5 min, 3 and 7 h, the leaves were removed and rinsed gently with distilled water for 30 s. To enumerate the attached bacteria a single 2 cm diameter disc was cut from a portion of each leaf in an area lacking major veins using a cork borer, the discs homogenized using a mortar and pestle, and cells enumerated by dilution plating on NYGA as in other studies (Chatnaparat et al., 2012). The experiments were repeated three times.

Motility analysis

Fresh colonies of an Xag wildtype, a *hem* mutant, and a complemented *hem* mutant from NYGA plates were stabbed into swarm and swimming plates composed of 0.03% (wt/vol) Bacto Peptone, 0.03% yeast extract, and 0.4% agar for swarm plate and 0.25% agar for swimming plate respectively. The inoculated cells were cultured for four days or longer at 28°C and examined for bacteria motile away from the inoculated site (Sockett and Armitage, 1991). The experiments were repeated three times and each experiment was measured in triplicate.

Extracellular enzymes assay

Relative levels of extracellular production including carboxymethyl-cellulase, α -amylase and protease were assessed by radial diffusion assays (Thowthampitak et al., 2008). The experiments were repeated three times and each experiment was measured in triplicate.

For carboxymethylcellulase production, inoculated plates containing an assay medium (0.1% carboxymethyl cellulose, 25 mM sodium phosphate, pH 7.0, and 0.8% agarose) were incubated at room temperature overnight, stained with 0.1% Congo red for 20 min, and washed twice with 1 M NaCl. Carboxymethyl cellulase (CMCase) activity was visualized as white halos surrounding the wells.

For α -amylase production, inoculated plates containing alpha-amylase assay medium (0.5% yeast extract, 1.0% tryptone, 0.25% NaCl, 0.2% soluble starch, and 0.8% agarose) were incubated at room temperature overnight and stained with potassium iodide for 10 min. α-amylase was detected as clear halos surrounding the wells.

For protease production, inoculated plates containing NYGA supplemented with 0.5% skimmed milk were incubated at room temperature for 48 h. Extracellular protease production was detected visually as clear halos surrounding the wells.

Hypersensitive response (HR) and virulence assay

The *X. axonopodis* pv. *glycines* strains were grown in NYGB at 28°C with shaking at 200 rpm. Cells were pelleted at early log phase by centrifugation at 6,000 rpm for 2 min. Cell pellets were suspended in sterile water for HR tests on tobacco. HR was assayed as described previously (Kaewnum et al., 2005). Briefly, tobacco plants were inoculated with bacterial suspensions (10^9 cells/ml) by injection of leaf with a syringe. Sterile demineralized water was used as a negative control. Infiltrated zones were observed for development of typical HR (tissue collapse and necrosis) for 24 to 48 h post-infiltration. The experiments were repeated three times with similar results.

The virulence of *X. axonopodis* pv. *glycines* strains was assessed on susceptible soybean cv. Spencer following topical spray application (Kaewnum et al., 2005). Briefly, cell suspensions of a given strain (OD_{600} = 0.2; ca. 10^8 cells/ml) in 1 mM KPO_4 buffer were sprayed onto leaves of plants (ca. 6 weeks old) maintained in a greenhouse (average temperature ca. 28°C). For the first 24 h after inoculation, plants were held in an enclosed plastic bag to maintain high humidity and moisture on leaves before being returned to the greenhouse bench. Three trifoliate leaves, collected each from the top, middle and basal portion of three plants from each of five replicate pots, were evaluated for each strain.

Cotyledon assay was done as described by Hwang et al. (1992). The 7-days old soybean seedling grown in a greenhouse was surface sterilized with 0.5% sodium hypochloride for 3 min and washed with sterile distilled water for 5 min. The cotyledon was punctured with sterile pins. 10 μl of each suspension of bacterial cells (10^8 cells/ml) of the wildtype, *hem* mutant, and complemented *hem* mutant were dropped on the wound site. Inoculated cotyledons were kept in high moisture conditions with 16h photo period at room temperature. The cotyledons were observed by chlorotic and necrotic symptoms around the inoculation site within 48 h after inoculation. At least five soybean cotyledons were used for each strain. The experiments were repeated three times with similar results.

Bacterial population on soybean leaf surface

For determination of epiphytic fitness, bacterial populations were isolated from the soybean leaves according to the modify method described by Morris et al. (1998). Inoculation of *X. axonopodis* pv. *glycines* strains on 6 weeks old soybean were designed to analyze the epiphytic fitness of the *hem* mutant in comparison to that of the Xag wildtype. Bacterial cultures were prepared to a final concentration of 4.5×10^5 cells per ml and then 50 ml of bacterial suspension was used to spray on soybean leaves as described above in virulence assay. Four pots containing 5 soybean plants in each pot were used for each strain. The plants were transferred to the greenhouse bench. Four leaves of inoculated soybeans were taken randomly from each treatment. Estimation of each bacterial numbers of the Xag wildtype, *hem* mutants, and complemented *hem* mutants were collected at 1, 3, 7 and 14 days after inoculation. The experiments were repeated two times.

RESULTS

Genetic characterization of the *hem* locus

Analysis of the DNA sequence of *X. axonopodis* pv. *glycines* 12-2 draft genome (GenBank accession number AJJO01000000) revealed the presence of the genes predicted as hemin uptake (*hem*) locus including hemin uptake protein, hemin uptake system outer membrane receptor, and hemin transport protein (*hem*), respectively. *hem* size is 800 bp encodes a protein of 211 amino acids.

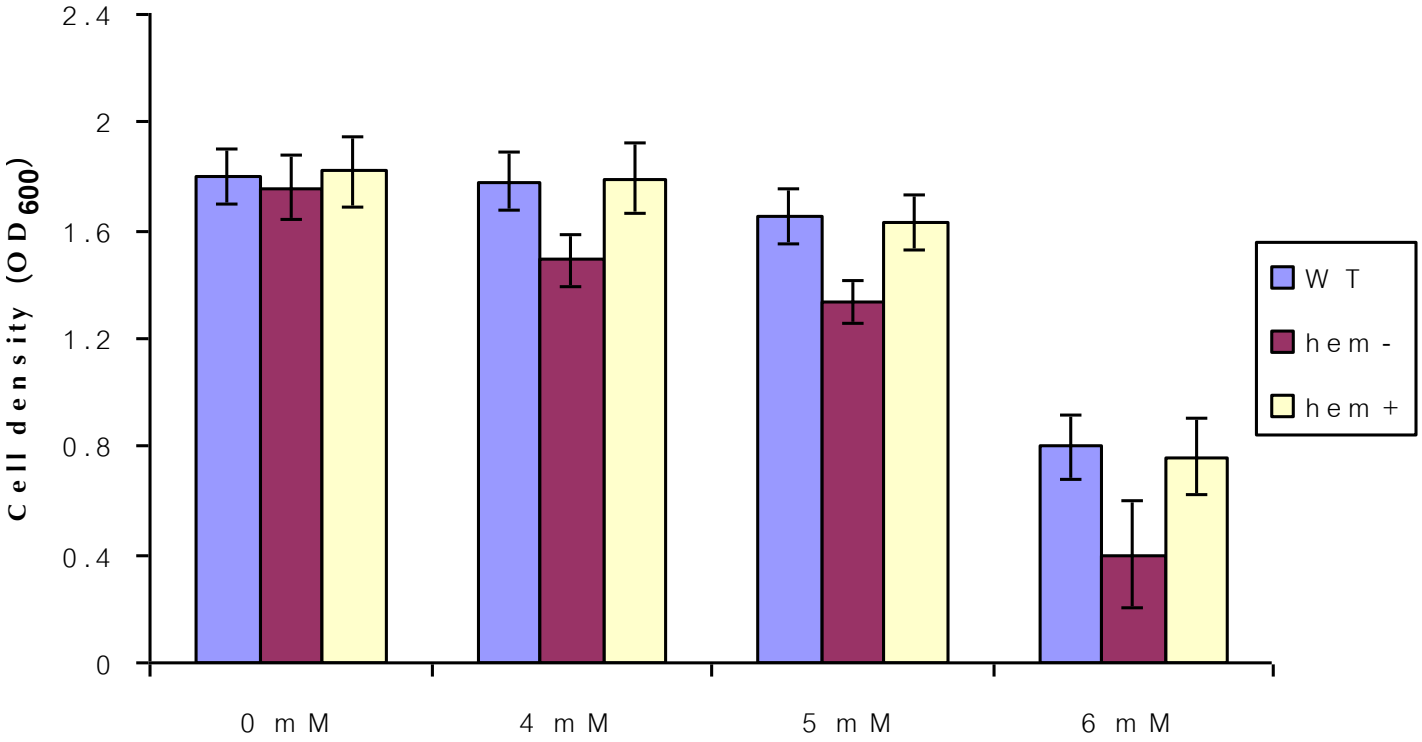

Figure 2. The growth of *Xanthomonas axonopodis* pv. *glycines* strains under different $FeCl_3$ concentration. Culture (10 µl, cell density adjusted to about 10^8 cells/ml) of each strains was inoculated into 5 ml of NYGB supplemented with different concentration of Fe^{3+} and were incubated at 28°C in shaker. WT = wildtype, *hem*$^-$ =*hem* mutant, and *hem*$^+$ = complemented *hem*. The cell density was measured spectrometrically at 600 nm after incubation for 24 h. Bars represent standard error of the means.

Hem shared the highest level of identity (99%) to the hypothetical protein of *Xanthomonas axonopodis* pv. *citri* 306 and *Xanthomonas citri* pv. *mangiferaeindicae* LMG 941, while it exhibited 80 and 70% identity to a hypothetical protein in *X. campestris* pv. *vesicatoria* 85-10 and *X. campestris* pv. *campestris,* respectively. However, protein predicted as hemin transport protein in *X. axonopodis* pv. *glycines* 12-2 shared similarity at 63 and 37% with hemin transport protein in *Stenotrophomonas maltophilia* D457 and *Sinorhizobium fredii* HH103 respectively.

High iron concentration sensitivity

The *X. axonopodis* pv. *glycines* wildtype and the *hem* mutant showed the same growth yield in NYGB without supplementation of $FeCl_3$. When NYGB supplemented with $FeCl_3$ to final concentrations of 0, 4, 5, and 6 mM, respectively, significant differences in growth were observed between the Xag wildtype and the *hem* mutant. The Xag wildtype could grow well in NYGB supplemented with concentrations up to 5 mM, whereas the *hem* mutant decreased the growth under all conditions (Figure 2). The growth capacity of the *hem* mutant could be completely restored in the complemented *hem* mutant.

Pathogenicity and virulence assay

Leaf pathogenesis assay was conducted to determine the probable involvement of *hem* functions in bacterial virulence. The *X. axonopodis* pv. *glycines* wildtype strain, the *hem* mutants, and the complemented *hem* mutant were inoculated through injection to soybean cotyledons and through spray on soybean leaves. All strains developed normal disease symptoms as in its wildtype when inoculated through injection into the soybean cotylendons (Figure 3B). Interestingly, the virulence of the *hem* mutant appeared to be attenuated when the cells were applied on soybean leaves by spray inoculation (Table 3, Figure 3A). Moreover, the hypersensitive response activities of the *hem* mutant were similar to that of the Xag wildtype after infiltration into tobacco. This results suggest that *hem* gene is essential for virulence of *X. axonopodis* pv. *glycines* on soybean before penetrate into soybean plant. Thus, perhaps *hem* gene is important for epiphytic fitness of this

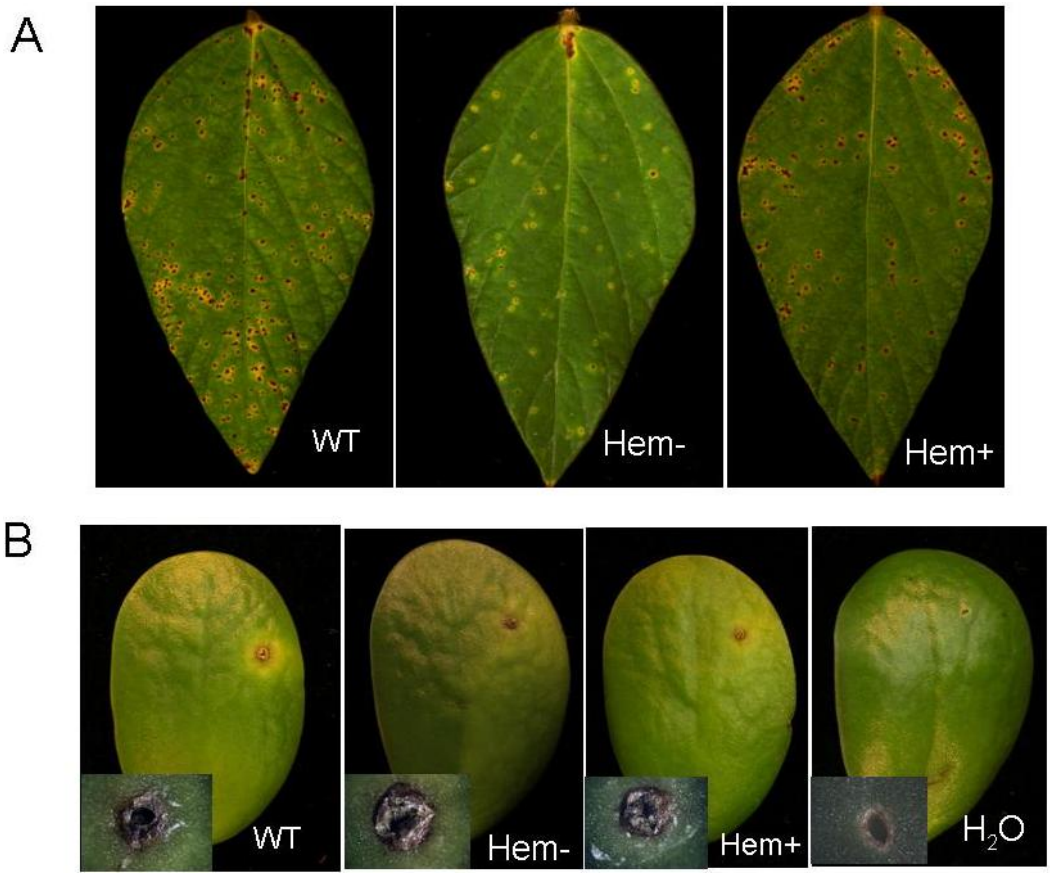

Figure 3. Virulence testing of the *Xanthomonas axonopodis* pv. *glycines* strains when bacterial cells were sprayed on soybean (A) and were injected into soybean cotyledons (B). WT = wildtype, *hem*$^{-}$ = *hem* mutant and *hem*$^{-}$ = *hem* complementary strain.

Table 3. Virulence deficient of *Xanthomonas axonopodis* pv. *glycines* by spray inoculation.

Strain	Mean of lesions per plant[a]	Relative virulence (%)
Xag12-2	260 ± 75	100
hem mutant	65 ± 23	25
hem complementary strain	246± 54	94

[a]Data shown are the averages ± standard deviations.

this pathogen.

hem gene expression

Real-time qRT-PCR was performed to determine transcript levels of *hem* gene in the *X. axonopodis* pv. *glycines* wildtype and the *hem* mutant in overnight culture in NYGB and also wildtype in soybean leaves after four days inoculation. Mutation in *hem* resulted in no expression of *hem* transcription in the culture medium confirming that the *hem* mutant strain completely lost *hem* gene. Furthermore, the level of *hem* transcript in the Xag wildtype cells recovered from infected soybean plants was 4.34 fold lower than that in the wildtype cells grown in culture (Figure 4). In addition, the expression of *hem* gene was not observed from cDNA of non-infected soybean. However, disruption of the *hem* gene did not affect the expression of the *hrpF* and *hrpD* genes, which are encoded in the *hrp* cluster when comparison with wildtype cells grown in Hrp inducing medium (data not shown). This results suggest that *hem* gene was down-

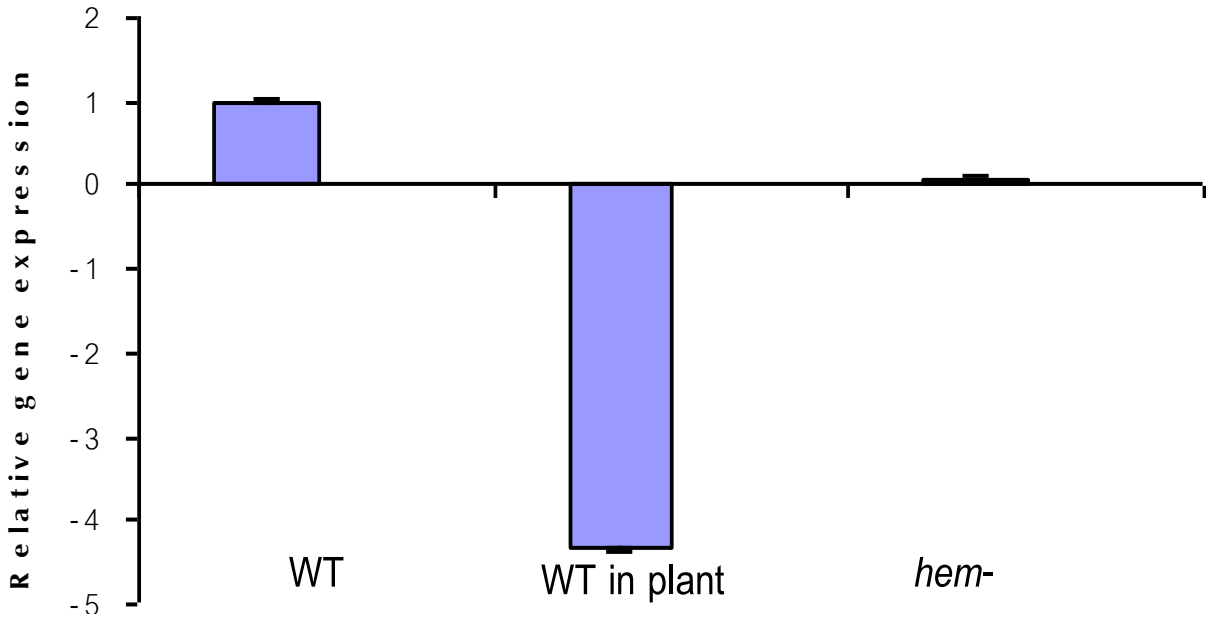

Figure 4. Relative abundance of transcripts of *hem* gene in *Xanthomonas axonopodis* pv. *glycines* wildtype (WT) and *hem* mutant (hem$^-$) grown in culture medium compared with wildtype in soybean plant as determined by real-time qRT-PCR. Vertical bars represent the standard error of mean ratio.

regulated when bacteria grow in soybean plant and did not affect typeIII secretion system of this pathogen in *vitro*. Therefore, the *hem* mutants show a virtually indistinguishable disease symptom when compared to that of the wildtype strain when the cells were directly injected into soybean (by pass epiphytic fitness phase).

Bacterial population on soybean

The above data suggest that *hem* gene contributed virulence on soybean leave when spray inoculation but not when injected into soybean. Therefore, we assessed the ability of the *hem* mutants to grow on soybean leaf surfaces. As we expected, minimal cells number were observed in soybean leaves sprayed with the *hem* mutants (Figure 5). Taken together, these data indicate that *hem* gene is required for the leaf colonization of Xag on soybean.

Extracellular polysaccharide (EPS) production

The EPS production in three-day liquid cultures showed that *hem* mutants produced on average 0.62 mg per milliliter of the culture, compared with 1.0 and 1.1 mg per milliliter of the culture of wildtype and complemented *hem* mutants, respectively (Figure 6). EPS is an important virulence factor in *X. axonopodis* pv. *glycines* and in many pathogens (Braun, 1990; Thowthampitak et al., 2008). Our results showed the *hem* mutants decreased the production of EPS. Thus, we assumed that the reduction in the production of EPS may contribute to the deficiency in virulence of the mutant.

Biofilm formation

Biofilm formation is structure for protect the bacterial cell from stress environmental condition. Plant pathogenic bacteria within biofilms are generally better resistant to environmental stress and host defense response (Crossman and Dow, 2004).The biofilm formation of the *hem* mutants in NYGB as measured by crystal violet staining after 3 days of incubation was significantly lower than that of the wildtype and complemented *hem* mutants (Figure 7). Therefore, the *hem* mutants may not resistant to environmental stress such as dry conditions or UV and host defense response during pathogenesis.

Bacterial attachment

Since we found that *hem* gene effected to biofilm formation on abiotic surface. Therefore, the attachment of *hem* mutants to the soybean leaves surface was studied by quantifying those cells remaining on leaves after they were dipped into bacterial cell suspensions. The number

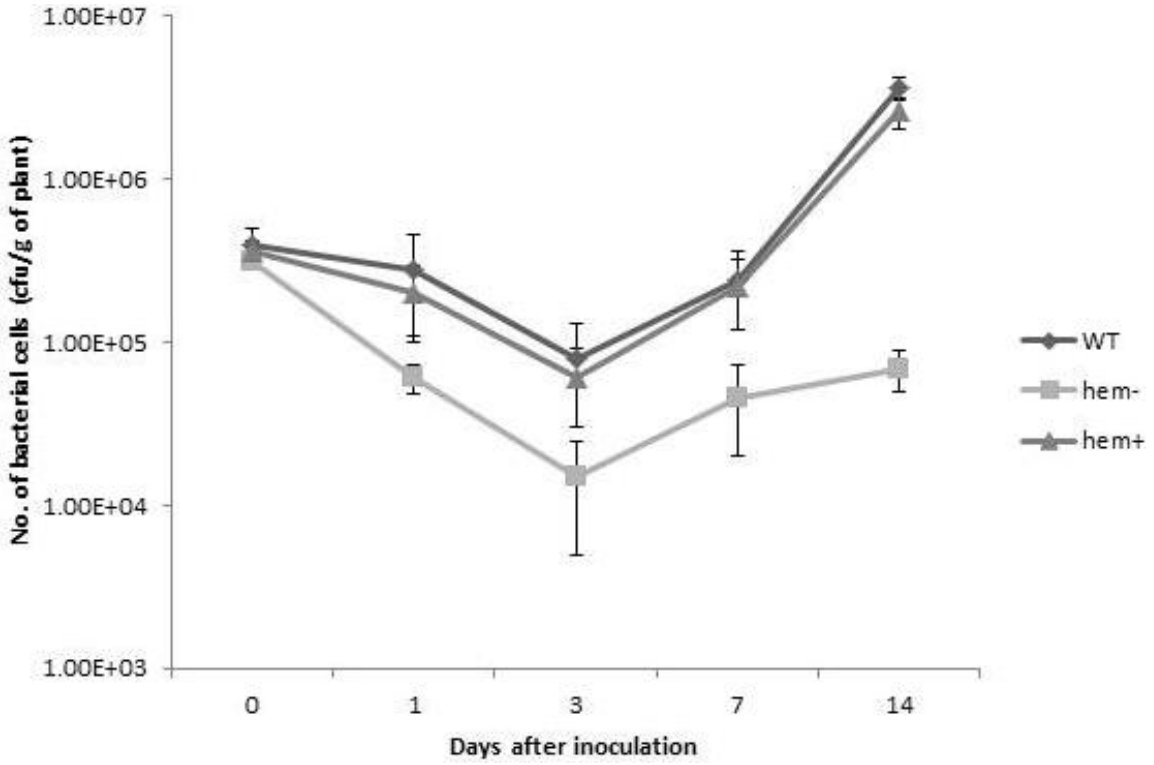

Figure 5. Bacterial population of *Xanthomonas axonopodis* pv. *glycines* strains on soybean leave surface after inoculation of each strain, at a concentration of 4.5×10^5 cells ml^{-1}, onto soybean leaves in a greenhouse experiment. WT = wildtype, *hem*$^-$ = *hem* mutant and *hem*$^-$ = *hem* complementary strain.

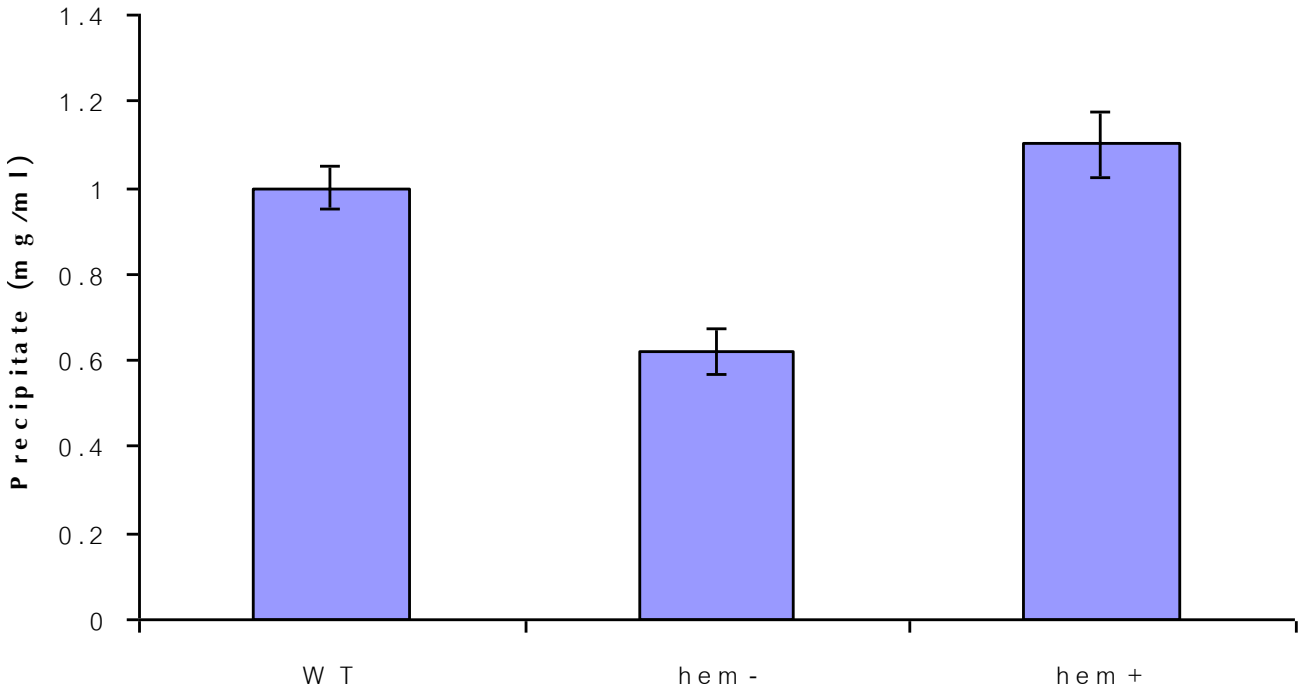

Figure 6. The *hem* mutant of *Xanthomonas axonopodis* pv. *glycines* affects the production of extracellular polysaccharide (EPS). WT = wildtype, *hem*$^-$ =*hem* mutant, and *hem*$^+$ = complemented *hem*. Bars represent the standard error of the means.

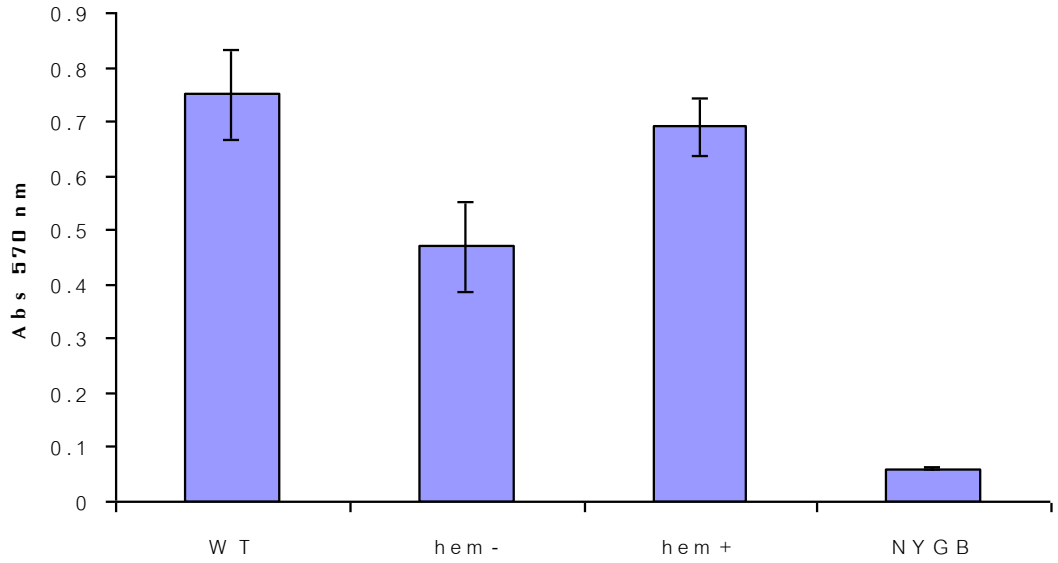

Figure 7. Biofilm formation on glass tube surfaces were assessed by the use of absorbance; WT = wild-type, *hem*$^{-}$ =*hem* mutant, *hem*$^{+}$ = complemented *hem*, and NYGB = liquid medium only.

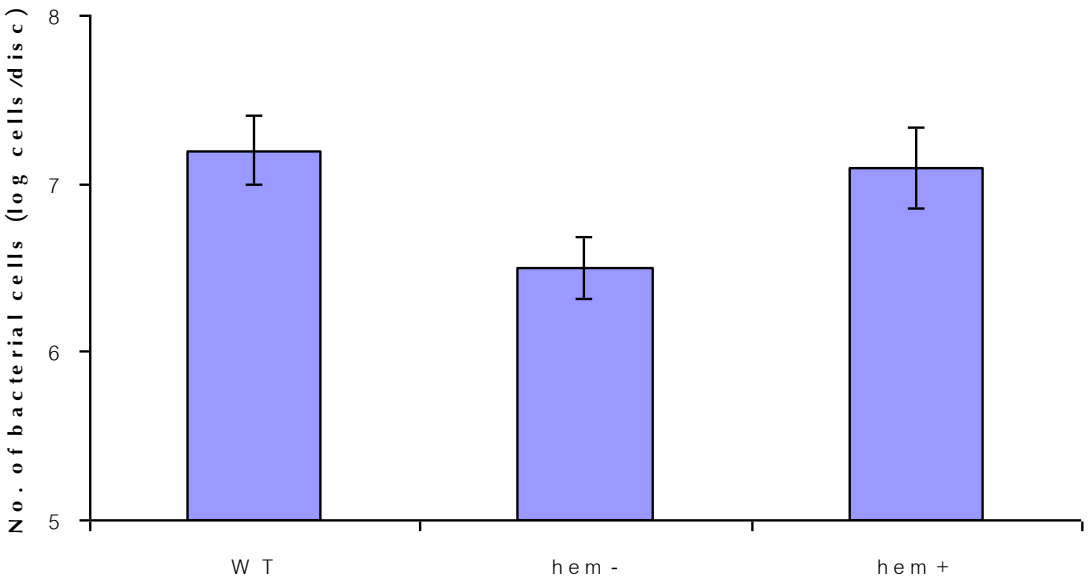

Figure 8. Attachment of the *Xanthomonas axonopodis* pv. *glycines* strains to the surface of soybean leaves after immersing individual leaves into cell suspension of wildtype (WT), *hem* mutant (*hem*$^{-}$) and complemented *hem* strain (*hem*$^{+}$)(10^{7} cells/ml) at 28°C.

of *hem* mutants cells was attachted soybean leaves surface significantly lower than the wildtype and complemented *hem* mutants (Figure 8).

Motility analysis

The *hem* mutant cells were swarming motile on semi-solid medium with 0.4% agar same as the wildtype and complemented *hem* mutant. However, we found that the swimming ability of the *hem* mutants on 0.25% agar swimming plate was significantly reduced when compared with the wildtype and complemented *hem* mutants (Figure 9). This result suggests that the *hem* mutants have effect to swimming motility but not for swarming movement.

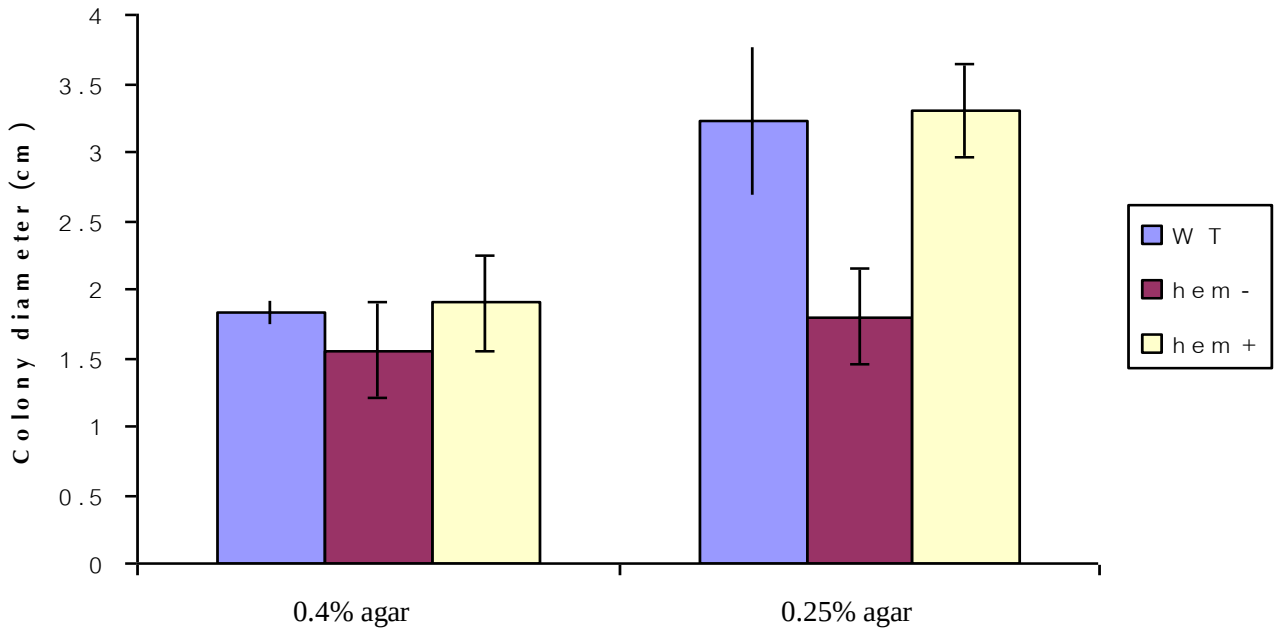

Figure 9. The motility zone of colonies grown in swimming and swarming plates with 0.25% and 0.4% agar respectively was measured at 2 days. WT = wildtype, *hem*$^{-}$ = *hem* mutant and *hem*$^{+}$ = *hem* complementary strain. Each data point is an average of 4 independent experiments, error bars indicate the standard error. The results are representative of four independent experiments.

Extracellular enzyme production

The production of extracellular proteases, amylases, and cellulase have been shown to be virulence factors in *X. axonopodis* pv. *glycines* 12-2 (Thowthampitak et al., 2008). To explore whether the *hem* mutant might also effects such virulence factors, these traits were compared among the wildtype, *hem* mutant, and complemented *hem* mutants in diffusion plate assay. We found that the expression of these extracellular enzyme productions did not differ among them (data not shown).

DISCUSSION

The acquisition of iron is thus one of the most important adaptive responses for bacterial pathogens. The ability of pathogenic bacteria to acquire iron from free heme and host hemoproteins has been studied by many laboratories for clinical pathogens (Braun et al., 1998; Lee and Levesque, 1997) but has not been reported in bacterial plant pathogens. We also found the sequence of hemin uptake locus system in draft genome of a bacterial pustule pathogen *X. axonopodis* pv. *glycines* 12-2 (GenBank accession number AJJO01000000). Sequence analysis of the hemin uptake locus revealed three genes including hemin uptake protein (Xag857), hemin uptake system outer membrane receptor (Xag858), and hemin transport protein (*hem*) required for use of hemin and hemoproteins as iron sources. In this study, we have analyzed the function of *hem* gene coding for hemin transport protein of *X. axonopodis* pv. *glycines* 12-2 that effect to virulence on soybean in the epiphytic phase of infection involving the extracellular polysaccharide (EPS) production, biofilm formation, attachtment, and motility. These might be suggesting that this *hem* system might be important for bacterial-plant interaction.

The colonization as epiphytic of *X. axonopodis* pv. *glycines* before infects through stomata or wounds on soybean leaves is very important process for cause pustule disease on soybean. In this report, the *hem* mutant of *X. axonopodis* pv. *glycines* was virulence deficient and decrease in the population size when sprayed on soybean plants but not when injected directly to soybean leaves. These results suggest that the hemin transport protein is essential in epiphytic phase, but not required for endophytic phase**.** Therefore, the reduction in virulence of the *hem* mutant is possible that *hem* might affect the expression of genes involving in the epiphytic fitness. Previous reports have indicated that *hem* is a multifunctional regulatory protein which controls the expression of trypsin-like protease, hemagglutinating, and hemolysin activities, as well as the production of extracellular vesicles in *Porphyromonas gingivalis* (Carman et al., 1990). Disruption of the hemin transport protein would result in the effected expression of these factors in *X. axonopodis* pv. *glycines*. This hypothesis is consistent with the regulation of hemin-responsive genes

in bacteria by a negative regulator such as the well-described *E. coli* ferric uptake regulator (Fur). Fur acts as a classical negative regulator and uses Fe^{2+} as a core-pressor to bind the promoter region of iron-regulated genes (Bagg and Neilands, 1987). Regulation of iron-regulated genes by a Fur-like system has been found in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* plants (Jittawuttipoka et al., 2010; Subramoni and Sonti, 2005). The mutations in ferric uptake regulator were also resulted in the reduction in virulence of *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* on their host plants (Jittawuttipoka et al., 2010; Subramoni and Sonti, 2005).

In this study, the transcription level of *hem* was reduced when bacteria grow in soybean plant. After invasion into the leaf through stomata, bacteria multiply within the substomatal chambers and intercellular spaces of the spongy mesophyll (Jones and Fett, 1985). In the plant cells, iron concentration was higher than in the surface of the plant. Many iron and heme transport systems are repressed by iron and Fur under iron-rich conditions (Lee, 1995). A component of an iron-scavenging system (PSPTO2134) of *P. syringae* pv. *tomato* DC3000 appears to be repressed under high iron concentration conditions (Jones and Wildermuth, 2011). While virulence control by iron has been well illustrated for the Fur system in *P. aeruginosa*, the Fur system is a negative regulator, indicating that the system represses the uptake of iron when iron is rich (Lamont et al., 2002). Furthermore, expression analyses in *P. syringae* pv. *tomato* DC3000 cultures indicates that high iron [50 μM iron(III) citrate] both represses high-affinity iron-scavenging system expression and induces expression of the type III secretion system and virulence genes in culture (Jones and Wildermuth, 2011).

From our data it seems that *hem* was suppressed in the high iron concentrations at 6 mM and in the plant cells which assumed is an also high iron concentration. Therefore, *hem* mutant shows the disease severity as a wildtype when inject the cells directly to soybean plant. Other pathogens that colonize similar plant environments (that is, the leaf apoplast and vasculature) might be expected to be similarly. The addition of bean leaf apoplastic fluid to *Pseudomonas syringae* pv. *phaseolicola* NPS3121 grown in minimal medium resulted in the expression of virulence genes and the repression of high-affinity iron import systems (Hernández-Morales, 2009). It was expected that abundant iron would repress high-affinity iron scavenging, but the induction of virulence genes was surprising. In a follow-up study, Kim et al. (2009, 2010) found that expression of virulence factors of *Pseudomonas syringae* pv. *tomato* DC3000 in *hrp*-inducing minimal medium was limited by iron availability and that higher iron to well above 10 μM continued to induce higher virulence gene expression. Indeed, we observed that the *hem* mutant did not affect type III genes expression under *hrp*-inducing minimal medium, this result suggesting that type III secretion system may be involved via a mechanism independent of the *hem* gene. For growth in *vitro*, the *hem* mutant exhibits an increase in sensitivity to iron when grown in the high iron media, compared to the wildtype. The growth effect of *hem* mutant under high iron concentration might be due to iron catalyzes the Fenton reaction as in Fur and Zur systems. This growth defect phenotype also found in the *fur* and *zur* mutants in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* respectively (Jittawuttipoka et al., 2010; Tang et al., 2005; Yang et al., 2007). The *zur* mutant was also exhibited an increase in sensitivity to zinc or iron when grown in the high zinc or iron media compared to the wildtype in *X. oryzae* pv. *oryzae* (Yang et al., 2007). In case of *P. syringae* pv. *tomato* DC3000, the iron-rich condition is around 200 μM and iron toxicity begins at over 400 μM. The toxicity was expected because iron catalyzes the Fenton reaction, producing the highly reactive hydroxyl radical, that result in reduced aerobic growth (Andrews et al., 2003). Moreover, the haem-uptake gene cluster in *Vibrio fischeri* is also regulated by Fur and contributes to symbiotic (Septer et al., 2011).

The successful establishment of a pathogen within a specific niche requires the ability of the pathogen to sense the specific environmental conditions of the host and to regulate the expression of virulence genes accordingly (Mekalanos, 1992). It is interesting to speculate that in response to hemin limitation, *X. axonopodis* pv. *glycines* is capable of turning on the expression of several factors which appear to be involved in the virulence potential of this organism. In this study report that *hem* was affected to the EPS production, biofilm formation, attachment, and motility but did not for extracellular enzymes production. This inference is consistent with the observations of hemin uptake system to enhanced expression of several putative virulence factors by *Streptococcus pneumoniae* and *Porphyromonas gingivalis* in mammalian hosts (Tai et al., 1993; Genco et al., 1995). Similar to the *zur* gene, the *X. oryzae* pv. *oryzae zur* mutant decreased the production of EPS and virulence on rice (Yang et al., 2007).

Previous report indicated that the existence of significant amounts of iron in corn seeds affected in bacterial adhesion and host colonization processes (Jacobs and Walker, 1977). The effect of hemin system to a role in EPS synthesis, and reduction in biofilm formation has also been observed in hemin transport protein mutants in *Yersinia* (Jarrett et al., 2004).

Importantly, we also found that the *hem* mutant was deficient in adhesion to both abiotic surfaces and soybean leaf surfaces. A clinical study has shown that iron depletion alters the cell surface property of pathogenic bacteria and lowers their attachment to sur-faces (Harjai et al., 1996). It is therefore possible that iron limi-

tation reduces the ability of the *hem* mutant to attach to soybean leave leading to a lower colonization rate. In contrast with *P. gingivalis*, the decreased transport of hemin by *P. gingivalis* results in the increased expression of hemolytic and trypsin-like protease activities that may contribute to the enhanced invasiveness exhibited in the mouse subcutaneous chamber model (Genco et al., 1995). Moreover, we also found that the swimming ability of the *hem* mutant was reduced. For marine bacteria that specialize in living on particles and aggregates, the population swimming speed of marine bacteria were significantly reduced in no-iron treatment. This reduction in population swimming speeds resulted in lower diffusivity and subsequently a lower colonization rate (Tang and Grossart, 2007).

We were surprised to find that the level of extracellular enzyme productions was not differed in the *hem* mutant compared with the Xag wildtype. Since changes in quorum sensing are associated with varying iron levels and quorum sensing of Xag controls a variety of traits including extracellular enzymes production, EPS production, motility, and biofilm formation that contribute to the virulence and epiphytic fitness (Thowthampitak et al., 2008). In *Xanthomonas campestris* pv. *campestris*, the strain deficient in exbD2, which encodes a component of its unusual elaborate TonB system, had impaired pectate lyase activity and caused no visible symptoms for defense on the non-host plant pepper (Vorhölter et al., 2012). It seems possible that extracellular enzyme productions of Xag may be regulated by other iron uptake pathway. Indeed, disruption of *hem* impairs the epiphytic fitness of Xag, as observed by a significant decrease in the population size of the mutants on soybean leaves compared to wild-type. It might thus be expected that, *hem* of *X. axonopodis* pv. *glycines* strongly effects to the ability of bacteria to EPS production, biofilm formation, swimming motility and thus lead to advantage for survival and colonization on leave surface, dispersal throughout the soybean plant, and start the new cycle of pustule disease.

ACKNOWLEDGMENTS

This research was supported by Center for Advanced Studies for Agriculture and Food (CASAF) a fellowship from Kasetsart University.

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