# *CarAB* in *Pseudomonas fluorescens* SP007s Reduces Symptoms on Soybean Caused by *Xanthomonas axonopodis* pv. *glycines* that Links to The Role of Multiple Genes

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## ABSTRACT

*Pseudomonas fluorescens* SP007s has been reported to successfully reduce symptoms of pustule disease (BP) caused by *Xanthomonas axonopodis* pv. *glycines* (Xag) with its complex metabolites. The possible role of *carAB* in strain SP007s that encoded carbamoylphosphate synthetase (CPSase) to degrade a diffusible signal factor (DSF) of Xag was assayed in this study. Mutation in *carAB* resulted in lost ability to produce CPSase and to inhibit DSF of Xag demonstrated by thin layer chromatography (TLC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), high performance liquid chromatography (HPLC), and agar plate assay. Mutant SP007s*carAB*$^-$ affected multiple gene expression encoding extracellular products that included secondary metabolites associated with biocontrol traits. Mutations in *carAB* caused a decreased expression of *phl*, *plt*, *phzE*, *pch* (corresponding to 2,4-DAPG, pyoluteorin, phenazine, and pyochelin biosynthesis), and *rpoS*; whereas *rpoS* mutant mediated a decreased production activity of *carAB* expression suggesting these gene regulation required a functional *carAB* genes; which the stationary-phase sigma factor, RpoS was also essential for the regulated system of CPSase biosynthesis. Mutants deficient in the biosynthesis of CPSase reduced less-severe symptoms than wildtype on soybean plant, whereas CPSase-overexpression strain and purified CPSase displayed a better reduction of BP symptoms suggesting the control ability depended CPSase mechanism of SP007s was affected by adequate levels of this enzyme biosynthesis. Mutations in *rpoS* significantly reduced BP symptom compared to *carAB* and *phl*&*plt*, whereas a double mutant strain deficient in the biosynthesis of antimicrobial metabolites 2,4-DAPG and pyoluteorin was less BP suppression than *carAB*, suggesting a counteracted mechanism was the evidence of biocontrol by SP007s. Taken together, we demonstrate that *carAB* is required for CPSase biosynthesis that can degrade DSF of Xag resulting in symptom reduction as one of biocontrol mechanism by SP007s. Induced expression of other related-genes indicates the complexity of *carAB*-dependent CPSase biosynthesis which the additional assay remains to be experimentally elucidated.

**Keywords:** Biocontrol, carbamoylphosphate synthetase, bacterial pustule disease, antimicrobial metabolite genes, *rpoS* regulated gene expression

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## INTRODUCTION

Bacterial pustule (BP) of soybean caused by *Xanthomonas axonopodis* pv. *glycines* (Xag), is one of serious diseases in Thailand and many other production areas worldwide especially under warm and humid climates. In Thailand, production of green soybean often faces a problem with several limitations including quality control and pesticide residue. The biological control is an alternative management that can be adapted for application replacing fungicides and insecticides especially at 40 to 50-day old plant of no chemical allowance. This period is a susceptible growth stage for BP infection (Prathuangwong, 2009). A biocontrol agent *Pseudomonas fluorescens* SP007s has been used to control various diseases in multiple crops including soybean (Hemsanit and Prathuangwong, 2009; Prathuangwong, 2009). However, the main mechanism that *P. fluorescens* SP007s exhibits ability to enhance plant health is still uncertain. A group of biocontrol *Pseudomona*s species generally composes biodiversity as plant growth promoting rhizobacteria (PGPR) that contributes beneficial effects on plant development through both mechanisms of biocontrol and biofertilization (Ahmad *et al*., 2011; Maheshwari, 2013; Siddiqui, 2006). The primary mechanism almost involves the production of extracellular metabolites, proteins, or a cascade of signal molecules such as antibiotics, iron chelating siderophores, cyanide and lytic enzymes (Nagarajkumar *et al*., 2004; Ramyasmruthi *et al*., 2012). *P. fluorescens* SP007s has been shown to produce a complex-antimicrobial compounds for plant growth promotion. The variety of such complexes whether formed independently of each other is remained to be fully described (Kladsuwan *et al*., 2013a, 2013b; Prathuangwong *et al*., 2005). The study directed a hypothesis if *carAB* in *P. fluorescens* SP007s produced carbamoylphosphate synthetase (CPSase) to degrade bacterial signal molecules of cell-to-cell communication and its extensive role might be across various antimicrobial activities.

A soybean BP pathogen, Xag and almost *Xanthomonas* species produce diffusible signal factor (DSF), a fatty acids signal molecule involved in a cell-to-cell communication for regulated mechanism named quorum sensing (QS) that modulate a population increase within microbial community (Ryan and Dow, 2008; Tang *et al*., 1991; Thowthampitak *et al*., 2008). Phytopathogenic *Xanthomonas* including *X. axonopodis* pv. *glycines* will not be able to aggregate population density for infection if they produce inadequate DSF (Barber *et al*., 1997; Thowthampitak *et al*., 2008). *X. axonopodis* pv. *glycines* produced DSF related to a well-characterized QS molecule produced by other *Xanthomonas* spp. (Thowthampitak *et al*., 2008). The *rpfF* gene from *X. axonopodis* pv. *glycines* strain 12-2 encoded the biosynthesis of DSF then, *rpfF* mutant lost ability to produce DSF. The mutant showed virulence reduction on soybean and produced less than wildtype levels of various exoenzymes and extracellular polysaccharides (EPS) demonstrating DSF is virulence factor of *X. axonopodis* pv. *glycines* (Thowthampitak *et al*., 2008). Control synthesis or degradation of the DSF cell-cell signal produced by plant pathogenic bacteria also caused defective biofilm formation and resulted in reduced virulence on their host plants (Chatnaparat *et al.,* 2012; Newman *et al*., 2008). We examined whether *carAB* in *P. fluorescens* SP007s produced CPSase that could degrade the DSF employed by Xag for its QS. Disruption or inactivation of QS regulation is called quorum quenching (QQ) that can disintegrate a bacterial population density-dependent attack (Chong *et al*., 2012; Pustelny *et al*., 2009; Zhang, 2003).

Several bacterial antagonists have been identified for cell-cell signal degradation via specific enzyme activities such as *Bacillus*, *Paenibacillus*, *Microbacterium*, *Staphylococcus*

and *Pseudomonas*. A particular enzyme named CPSase that catalyzed ATP dependent the synthesis of carbamoylphosphate from glutamine and ammonia, a precursor for pyrimidine and arginine biosynthesis encoded by *carAB* in various bacterial antagonists can rapidly degrade DSF produced by pathogenic *Xanthomonas* species (Newman *et al*., 2008; Tarighi and Taheri, 2011; Guy *et al*., 1998). CPSase activity is encoded by two genes, *carA* and *carB*. The small subunit of this enzyme complex, a glutamine amidotransferase encoded by *carA* where *carB* encodes and catalyzes the large subunit, CPSase (Kwon *et al*., 1994). The purpose of this work was then, to characterize the *carAB* genes responsible for encoding CPSase in *P. fluorescens* SP007s on biocontrol pathway against soybean BP caused by *X. axonopodis* pv. *glycines*. Identification of mutant that controlled expression across antimicrobial activities was also described. Antimicrobial activity employed by plant-associated *P. fluorescens* SP007 involving the ecological interaction of biocontrol and biofertilization traits has been recently reported (Prathuangwong *et al*., 2009). Data obtained provide a better understanding of biocontrol mechanism that in return, reach an ultimate goal of effective *P. fluorescens* SP007 application.

The *rpoS* (RNA polymerase, sigma S) is a sigma factors are proteins that regulate transcription in bacteria. Sigma factors can be activated in response to different environmental conditions. The *rpoS* is transcribed in late exponential phase, and RpoS is the primary regulator of stationary phase genes. RpoS is a central regulator of the general stress response and operates in both a retroactive and a proactive manner, it not only allows the cell to survive environmental challenges (Aronis, 2002). A correlation was found between amount of extracellular proteins and the repressed-genes cues. Data obtained suggest the involvement of CPSase produced by SP007s which demonstrate suppression BP. Potential interaction and association of *carAB* with decrease or increase of multiple extracellular protein syntheses are yet remained for most effective *P. fluorescens* SP007s application.

## MATERIALS AND METHODS

### Strains, Plasmids and Conditions

*P. fluorescens* SP007s and *X. axonopodis* pv. *glycines* 12−2 and the plasmids used in this study are listed in Table 1. Each strain was routinely cultivated on King's medium B agar (KB) and Luria-Bertani (LB) agar plates at 28°C and 37°C where appropriate. All DNA isolation and manipulations were performed as described previously (Sambrook *et al*., 1989). Media were amended with kanamycin (50 µg/ml) and gentamicin (15 µg/ml) for selection when required.

**Table 1** Bacterial strains and plasmids used in this study

| Bacterial strains or plasmid | Relevant characteristic[a] | Reference |
|---|---|---|
| ***Escherichia coli* Top10** | lacZΔM15, endA1, recA1, *hsd*R, mcrA | Invitrogen |
| Top10 (pTok2-*carA*) | *E.coli* Top10 carrying pTok2-*carA* | This study |
| Top10 (pTok2-*carB*) | *E.coli* Top10 carrying pTok2-*carB* | This study |
| Top10 (pTok2-*carAB*) | *E.coli* Top10 carrying pTok2-*carAB* | This study |
| Top10 (pTok2-*rpoS*) | *E.coli* Top10 carrying pTok2-*rpoS* | This study |
| Top10 (pBBR-*carA*) | *E.coli* Top10 carrying pBBR1MSC-5-*carA* | This study |
| Top10 (pBBR-*carB*) | *E.coli* Top10 carrying pBBR1MSC-5-*carB* | This study |
| Top10 (pBBR-*carAB*) | *E.coli* Top10 carrying pBBR1MSC-5- *carAB* | This study |
| Top10 (pBBR- *rpoS*) | *E.coli* Top10 carrying pBBR1MSC-5- *rpoS* | This study |
| Top10 (pTrp::*carAB*) | *E.coli* Top10 carrying pBBR1MSC-5- *carAB* gene fusion with trp promoter | This study |
| ***P. fluorescens* SP007s** | Biological control agent | Prathuangwong *et al.* (2005) |
| SP007s *carA*::Km$^r$ | *carA*::Km$^r$ of SP007s by gene deletion | This study |
| SP007s *carB*::Km$^r$ | *carB*::Km$^r$ of SP007s by gene deletion | This study |
| SP007s *carAB*::Km$^r$ | *carAB*::Km$^r$ of SP007s by gene deletion | This study |
| SP007s *rpoS*::Km$^r$ | *rpoS*::Km$^r$ of SP007s by gene deletion | This study |
| *carA*::Gm$^r$ (pBBR-*carA*) | *carA*::Gm$^r$ of SP007s carrying pBBR1MSC-5 | This study |
| *carB*::Gm$^r$ (pBBR-*carB*) | *carB*::Gm$^r$ of SP007s carrying pBBR1MSC-5 | This study |
| *carAB*::Gm$^r$ (pBBR-*carA*B) | *carAB*::Gm$^r$ of SP007s carrying pBBR1MSC-5 | This study |
| *rpoS*::Gm$^r$ (pBBR- *rpoS*) | *rpoS*::Gm$^r$ of SP007s carrying pBBR1MSC-5 | This study |
| *carAB*OX | SP007s wildtype, containing pTrp::*carAB* | This study |
| ***X. axonopodis* pv. *glycines* 12-2 Plasmid** | A causal pathogen of soybean bacterial pustule | Prathuangwong *et al.* (2004) |
| pBBR1MCS-5 | Broad host range cloning vector, *lacZ*, Gm$^r$ | Kovach *et al.* (1995) |
| pKD13 | FRT-Kmr-FRT, oriR6K, Ap$^r$, Km$^r$ | Densenko and Wanner (2000) |
| pTok2 | ColE1 replicon, suicide plasmid, Tc$^r$ | Kitten and Willis (1996) |
| pTok2:: Δ*carA* | Δ*carA*::Kan from overlapping PCR cloned into pTok2, Tc$^r$, Km$^r$ | This study |
| pTok2:: Δ*carB* | Δ*carB*::Kan from overlapping PCR cloned into pTok2, Tc$^r$, Km$^r$ | This study |
| pTok2:: Δ*carAB* | Δ*carAB*::Kan from overlapping PCR cloned into pTok2, Tc$^r$, Km$^r$ | This study |

**Table 1** (Continue)

| Bacterial strains or plasmid | Relevant characteristic[a] | Reference |
|---|---|---|
| pTok2:: Δ*phzE* | Δ *phzE*::Kan from overlapping PCR cloned into pTok2, $Tc^r$, $Km^r$ | This study |
| pTok2:: Δ*rpoS* | Δ *rpoS*::Kan from overlapping PCR cloned into pTok2, $Tc^r$, $Km^r$ | This study |
| pBBR:: *carA* | *carA* gene with native promoter cloned into pBBR1MCS-5, *lacZ*, $Gm^r$ | This study |
| pBBR:: *carB* | *carB* gene with native promoter cloned into pBBR1MCS-5, *lacZ*, $Gm^r$ | This study |
| pBBR:: *carAB* | *carAB* gene with native promoter cloned into pBBR1MCS-5, *lacZ*, $Gm^r$ | This study |
| pTrp::*carAB* | *carAB* gene fusion with trp promoter cloned into pBBR1MCS-5, *lacZ*, $Gm^r$ | This study |

[1/] $Km^r$ = kanamycin resistance, $Gm^r$ = gentamicin resistance and $Tc^r$ = tetracycline resistance

**Gene Knockout Mutagenesis**

The primer for amplification of *carA*, *carB* and *rpoS* genes in *P. fluorescens* SP007s were designed from genomic data (Table 2). Targeted disruption of *carA*, *carB*, double targeted disruption of *carAB* and *rpoS* genes in *P. fluorescens* SP007s were accomplished using overlap extension mutagenesis and sequenced to confirm their identity as previous described in Datsenko and Wanner (2000). Clones were selected on LB agar amended with kanamycin. Confirmation of the plasmid constructs were accomplished by sequence of PCR product from F1 and R2 primers (Table 2). In addition, the pTok2::Δ*carA*, pTok2::Δ*carB*, pTok2::Δ*carAB* and pTok2::Δ*rpoS* were transformed in to strain SP007s and transformants were selected on KB agar containing kanamycin for *carA*, *carB*, *carAB* and *rpoS* mutants. Confirmation of clones were subsequently verified for kanamycin genes fragment insertions by PCR with specific primers (FRT-Km-FRT-F and FRT-Km-FRT-R) and amplification mutant strain (Δ*carA*, Δ*carB,* Δ*carAB* and Δ*rpoS*) with internal primer (Table 2).

**Table 2** Polymerase chain reaction primers

| Primer | Sequence | Description |
|---|---|---|
| TAL2a-F | 5' TCACTGAGGCAATAGCTCCATC 3' | Amplification of 3,261 bp of TAL2a |
| TAL2a-R | 5' ATGCGGACTCGGAAGACGTA 3' | ..... |
| SP007s *hrpA*-F | 5' ATCATCACCTCGGCGACCAT 3' | Amplification of 500 bp of *hrpA* |
| SP007s *hrpA*-R | 5' ACCTTGGCGCGGTAGCTGTA 3' | ..... |
| *carA*-F | 5' TGAAAGCCAACGGCACCGT 3' | Amplification of 517 bp of *carA* |
| *carA*-R | 5' AACTGATGGCCGAGGCAGAT 3' | ..... |
| *carA*-KO-F1 | 5' ATGCGACGTATAGCCGTAGTG 3' | Amplification of upstream region (1,008 bp) of *carA* for generate *carA* mutant |
| *carA*-KO-R1 | 5' GAAGCAGCTCCAGCCTACACAGTATGGCTGG CTTAGTCAAGA 3' | ..... |
| *carA*-KO-F2 | 5' GGTCGACGGATCCCCGGAATTGTTCGACCGC TTCATCTC 3' | Amplification of downstream region (1,060 bp) of *carA* for generate *carA* mutant |
| *carA*-KO-R2 | 5' ACGCCGAACTTCTCCAGCA 3' | ..... |
| *carA*comF-*Bam H*I | 5' AGTAGGATCCTCCAGGAAGTGGCGGTCTA 3' | Amplification of 1,690 bp of *carA* for generate *carA* complementary strain |
| *carA*comR-*Bam H*I | 5' AGTAGGATCCAAGTCGCGAGGACGAACAAC 3' | ..... |
| *carB*-F | 5' ATGCCAAAACGTACAGACAT 3' | Amplification of 1,140 bp of *carB* |
| *carB*-R | 5' GACTTCATCTGGGTGGTCA 3' | ..... |
| *carB*-KO-F1 | 5' CGAATTCCTCGATACCGAG 3' | Amplification of upstream region (1,082 bp) of *carB* for generate *carB* mutant |
| *carB*-KO-R1 | 5' GAAGCAGCTCCAGCCTACACAGGATGCTCT TTATGTCTGTACG 3' | ..... |
| *carB*-KO-F2 | 5' GGTCGACGGATCCCCGGAATGATCTGTGAG GCGCTCAAG 3' | Amplification of downstream region (982 bp) of *carA* for generate *carB* mutant |
| *carB*-KO-R2 | 5' TCCCGACCTGGTTCAGAC 3' | ..... |

**Table 2** (Continue)

| Primer | Sequence | Description |
|---|---|---|
| *carB*comF-*Bam H*I | 5' AGTAGGATCCGTTGTTCGTCCTCGCGAC TT 3' | Amplification of 3,943 bp of *carB* for generate *carB* complementary strain |
| *carB*comR-*Bam H*I | 5' AGTAGGATCCGTTTTCCTTGAGGTCGCC CA 3' | ..... |
| *rpoS*-F | 5' CATGTGGTCAAGGAGCTCAA 3' | Amplification of 202 bp of *rpoS* |
| *rpoS*-R | 5' TATCCAGCAGGGTCTTGTCC 3' | ..... |
| *rpoS*-KO-F1 | 5' CTCGACTCGGTACGTTTCGT 3' | Amplification of upstream region (1,118 bp) of *rpoS* for generate *rpoS* mutant |
| *rpoS*-KO-R1 | 5' GAAGCAGCTCCAGCCTACACACTCCGGC CCTTCTTTTTTGA 3' | ..... |
| *rpoS*-KO-F2 | 5' GGTCGACGGATCCCCGGAATTTCGTCAG ATCCAGGTCGAG 3' | Amplification of downstream region (1,124 bp) of *rpoS* for generate *rpoS* mutant |
| *rpoS*-KO-R2 | 5' TGTCGGTGACGACGAAGGT 3' | ..... |
| rpoScomF-HindIII | 5' AGTAAAGCTTATCGGCCGTTTTGCCTCA 3' | Amplification of 1,542 bp of *rpoS* for generate *rpoS* complementary strain |
| rpoScomR-HindIII | 5' AGTAAAGCTTTACTTAGGCTCACACGCCGT3 ' | ..... |
| Ptrp-F-1 | 5' TAGTTAACTAGTACGAAAGTTCACATGAGAA GGAGGACAGCTTCTAGATCCAGGAAGTGGCG GTCTA 3' | For first round of placing trp operon promoter upstream of start codon of *carA* gene for construct *carA*-overexpression strain |
| Ptrp-F-2 | 5' GGCAAATATACTGAAATAGGTGTTGACATTAT TCCATCGAACTAGTTAACTAGTACGAAAGTTC 3' | For second round of placing trp operon promoter upstream of *carAB* this primer overlapping with Ptrp-F-1 |
| Ptrp-F-blunt | 5' TAAAGTTATGTCATGTACATCATAACGGTT CCGGCAAATATACTGAAATAGGTGTTGAC 3' | For third round of placing trp operon promoter upstream of *carAB* this primer overlapping with Ptrp-F-2 |

**Table 2** (Continue)

| Primer | Sequence | Description |
|---|---|---|
| overcarAB-R-blunt | 5' GTTTTCCTTGAGGTCGCCCA 3' | Amplification of *carAB* gene for construct *carAB* overexpression strian |
| FRT-Km-FRT-F | 5' GTGTAGGCTGGAGCTGCTTC 3' | Amplification of 1,304 bp of kanamycin gene |
| FRT-Km-FRT-R | 5' ATTCCGGGGATCCGTCGACC 3' | ..... |
| Gm-F | 5' ATGTTACGCAGCAGCAACGAT 3' | Amplification of 534 bp of gentamicin gene |
| Gm-R | 5' TTAGGTGGCGGTACTTGGGT 3' | ..... |

PCR-amplified fragment 1,690, 3,943, 5,613 and 1,542 bp (*carA*, *carB*, *carAB* and *rpoS*) containing native promoter using primer *carA*comF-*Bam H*I, *carA*comR-*Bam H*I, *carB*comF-*Bam H*I, *carB*comR-*Bam H*I, *rpoS*comF-*Bam H*I and *rpoS*comR-*Bam H*I, respectively and individual amplicons were digested with *Bam H*I and ligated into the multiple cloning site of vector pBBR1MSC-5 using T4DNA ligase (Thermo Fisher Sciencetific Inc., USA). Clones were selected on LB agar amended with gentamicin and X-gal for *carA*, *carB, carAB* and *rpoS*. Confirmation of the plasmid constructs (pBBR::*carA*, pBBR::*carB*, pBBR::*carAB*, and pBBR::*rpoS*) were accomplished by sequence of PCR and flanking each gene. The confirmed plasmid were then individually mobilized (pBBR::*carA*, pBBR::*carB*, pBBR::*carAB* and pBBR::*rpoS*) into Δ*carA*, Δ*carB*, Δ*carAB* and Δ*rpoS* by electroporation as described above. Complementary strains were selected on KB agar amended with gentamicin.

Characteristic of wild type, complemented and mutant of strain SP007s including growth rate, ability to inhibit *Xanthomonas axonopodis* pv. *glycines* 12−2 (Xag 12−2), motility, biofilm production, extracellular polysaccharides and extracellular protease productions were determined by plate count method, paper disc method (Chuaboon, 2008), motility agar (Lane *et al*., 2007), biofilm production test (O' Toole and Kolter, 1998), extracellular polysaccharides assay (Tang *et al*., 1991) and extracellular protease (Thowthampitak *et al*., 2008), respectively.

**Overexpression of *carAB* and DSF Biosensor**

The effect of QQ mechanism was confirmed with overexpression of *carAB* gene in *P. fluorescens* SP007s using the method described by Chatnaparat *et al.* (2012). The SP007s-overexpressing strain (SP007sOX) was constructed by placing the coding region of *trp* operon promoter and *carAB* gene into pBBR1MSC-5. *carAB* was amplified using *P. fluorescens* SP007s genomic DNA as the template with primers Ptrp-F-1, Ptrp-F-2, Ptrp-F-blunt, overcarAB-R-blunt (Table 2) and cloned into the *Sma*I site of pBBR1MSC-5 to yield pTrp::*carAB* by T4D ligase enzyme. The amount of recombinant plasmid was increased by transformed heat shock assay into *E. coli* TOP10. Selected colonies on LB amended X-gal and gentamicin. *carAB* constructs were done by PCR amplification and sequence analyzed. The cloning plasmid ligated with *carAB* genes was transformed into *P. fluorescens* SP007s competent cell was describe above. Transformants selected on LB agar containing

gentamicin and SP007sOX were confirmed done by PCR amplification with specific primers.

The DSF bioassay was carried out with biosensor *X. campestris* pv. *campestris* (pKLN55) that respond to exogenous QS by emitting bioluminesicense (Newman *et al*., 2004). Briefly, plasmid pKLN55 has a green fluorescent protein reporter gene (gfp) fused to DSF-inducible promoter region of *engXCA*. pKLN55 (*engXCA*::gfp) were transformed in to strain Xag 12-2 and transformants were selected on LB agar amended with streptomycin and spectiomycin. Confirmation resistance clones were visualized by Fluorescence Stereo Microscope (Leica M205 FA, Leica Microsystems, Germany).

**Extraction and Purification of CPSase**

Analysis of CPSase production was carried out after protein fraction of 24 h old culture supernatants (basal salts medium). CPSase activity was measured by TLC, SDS-PAGE, and HPLC, briefly proteins sample were spot on TLC plate coated with silica gel (Merck) with CPSase standard (catalog number MBS966352). The absorbing bands were examined under 254 nm UV light (Corvi *et al*., 2001). SDS-PAGE gels were run with 30 µl of protein sample from wildtype and mutants compared with standard according to Gam and Latiff (2005) and Chuaboon *et al*. (2014). Preparative HPLC was accomplished following the method described by Robino *et al*. (1987). The above proteins containing CPSase were subjected to HPLC. The CPSase was eluted with linear gradient HPLC spectra and detected by a UV monitor at 280 nm. The collected enzyme was used to compare with CPSase standard (catalog number MBS966352) and stored at -80°C.

Gene expression in coculture of SP007s-*carAB* (responded for CPSase biosynthesis) was also determined by qRT-PCR analysis that carried by using qPCR mix (Evagreen®, Solis biodyne). Total RNA was extracted from bacterial cells of each strain were collected and used for RNA extraction using RNeasy Mini Kit protocol (QIAGEN®) following the manufacture's instruction. To remove contaminating DNA were treated with Turbo DNA-free™ kit (Invitrogen, CA, USA), RNA samples were analyzed for quality using a nanodrop™ 2000 (Thermo Fisher Scientific Inc., USA). The cDNA was generated from 1 µg of RNA with RevertAid first stand cDNA synthesis kit (Thermo Fisher Sciencetific Inc., USA) and random hexamers. An internal standard was generated using purified *rpoD* gene (house keeping gene). To confirm that DNA was removed, samples processed in parallel without reverse transcriptase served as negative controls in qRT-PCR experiments. The transcriptional level of *carA*RT F: 5' TGATGATCACCAGCCAGA AC 3' and *carA*RT R: 5' GAGATGAAGCGGTCGAACAG 3'; *carB*RT F: 5' CCTGACCACCCAGATGAAGT 3' and *carB*RT R: 5' GAACACTTCCTCGACGCTCTG 3'; and *rpoD* F: 5' CTGCAATTCC TCGACCTGAT 3' and *rpoD* R: 5' TGAGCTTGTTGATCGTCTCG 3', primer amplification of 202, 241 and 196 bp fragments of genes, respectively.

**Assay of Quorum Sensing Disruption**

To evaluate the potential role of CPSase produced by SP007s in QS-degradation of DSF biosensor Xag 12−2 strain, Assay of inoculated-agar medium was investigated. The degradation of DSF was conducted using strains Xag 12−2 (pKLN55) and SP007s viability cells compared to Xag-viable cells added SP007s-CPSase. All bacterial strains were separately grown in LB medium for 48 hrs ($OD_{600nm}$ of 0.2), and the culture was used as an inoculum for NA plate assay. The 150 µl of a biosensor Xag 12−2 (pKLN55) was over-sprayed onto solid NA plate until well-spread (Linthorne *et al*., 2015) using a sterile airbrush (ABS1, Somax Co. Ltd, China). Each SP007s strain of 10 µl was then dropped onto NA plate center that earlier amended with Xag without allowing the test strains to grow. In a similar experiment, extracted CPSase was used

as inoculum instead of SP007s viability cells with varied-enzyme concentration to compare the DSF-degraded capacity. After 24 hrs incubation at 28°C, each inoculated plate was visually assessed under fluorescence stereo microscope for the evidence of brightly green fluorescence or darkness zones that indicated the presence or absence of DSF residue respectively.

**Analysis of Gene Expression and Involved Biosynthesis Products**

To confirm *carAB* regulated the production of some antimicrobial compounds, the expression of corresponding genes was determined using qRT-PCR using the method described above. The related genes selected including *phl* (2,4-DAPG), *plt* (pyoluteorin), *phzE* (phenazine), *pch* (pyochelin siderophores) and *rpoS* (sigma S). The qRT-PCR primers were *phl* F: 5' CCCGCCGACTATGACTATGT 3' and *phl* R: 5' GCCTGGTCCAGTACGTTGAT 3'; *plt* F: 5' GCGTCGATCTTTCAATCC AT 3'; *plt* R: 5' ACAGGCACCGTCGTGATT 3'; *phzE* F: 5' CTGAGTTTCT TCCGCCAGTT 3' and *phzE* R: 5' TC GAGGAATTCCATGACCTC 3'; *pch* F: 5' ACATGA ACGGCGATCTTCTC 3' and *pch* R: 5' ATCATCAGGCTCTGGCTG AC 3'; *rpoS* F: 5' CATGTGGTCAAGGAGCTCAA 3' and *rpoS* R: 5' TATCCAGCAGGGTCTTGTCC 3'. The biosynthesis products of different gene expression were determined by TLC and selective medium as appropriated.

Site directed mutation of target genes as described by Athinuwat *et al.* (2009) was conducted to support the coincidence of CPSase-dependent regulatory system against Xag infection. The primer for amplication of *phl* and *plt* genes in *P. fluorescens* SP007s were designed from genomic data (Phl- F-Up: 5' AGTAGAATTCTCGGACAAGAACAGCAT CG 3', Phl-R-Up: 5' AGTAGGCGCGCCCAT-GCTCCTTGCGTAGACA 3', Phl-F-Down: 5' AGTAGAATTCAGGAGCCCGGACCATG 3' and Phl-R-Down:5' AGTAGGCGCGCCGCTA-ACTTCAAGGTGCCTTATA 3'; and Plt-F-Up: 5' AGTAGAATTCAGCGATGTGCTGGCTCTCAA 3', Plt-R-Up: 5' AGTAGGCGCGCCATCAAC-GAGATGGCC AA GCGA 3', Plt-F-Down:5 'AG-TAGAATTCAAGGCTGAAGACCGTTCGC 3', and Plt-R-Down: 5 'AG TAGGCGCGCCAGA-TACCGAACTGCACGTTGGA 3'. Fragments containing the up and downstream sequences from *phl* and *plt* were individual ligated into pUC19 (Quick Ligation™ Kit, New England Biolabs). The kanamycin gene was amplified from EZ-Tn5™<KAN-2>Tnp Transposome™ (Epicentre, WI, USA) for selection in the *phl* mutants, whereas the gentamicin resistance gene (Kovach *et al*., 1995) was used for *plt* mutants. Confirmation of the constructs was accomplished by PCR and sequencing of pUC19:: Δ*phl* and pUC19:: Δ*plt*. To further elucidate the role of the specific genes, double mutants *phl*$^-$*&plt*$^-$ was generated by transformation with pUC19::Δ*plt* into SP007s*phl*$^-$ electroporation as previously described. Transformants were selected on KB agar containing kanamycin and gentamicin. The inhibitory effect against Xag growth by these production activities was determined by paper disc assay on NA plate (Kladsuwan *et al*., 2013b)

**Coinoculation on Soybean Plant**

Each bacterial strain of *P. fluorescens* SP007s were evaluated for biocontrol efficacy under greenhouse experiments. Soybean cv. SJ5 was planted as previously described (Prathaungwong *et al*., 1993). Each bacterial suspension of different SP007s strains were sprayed 6 hrs before Xag 12−2 (pKLN55) using standardized cell number as approximately as $1x10^8$ cfu/ml. Treatments were included coinoculation of Xag12−2 with strains SP007sOX, SP007s*carA*$^-$, SP007s*carB*$^-$, SP007s*carAB*$^-$, SP007s*phl*$^-$*&plt*$^-$, SP007s*rpoS*$^-$, SP007sWT, and its purified CPSase extract compared with Xag 12°2 alone inoculation, and dd$H_2O$ control treatment. The inoculated pots were incubated until the symptom development was visually scored (at 7 to 10 days after inoculation) upon

the percentage of holes on scoring card (4.0 ×7.0 cm) through which pustules were present (Prathuangwong *et al*., 1993). The biocontrol results of each strain ability were analyzed separately by analysis of variance and Duncan's multiple range test (DMRT) was used for separation of means ($P \leq 0.05$).

The determination of population dynamic of strains SP007s and Xag 12−2 on soybean leaves of inoculated plants were monitored during these experiments by collecting soybean leaves after bacterial inoculation. Leaf discs of 5 mm in diameter, were cut from leaves for up to 7 days post-infiltration. Samples were placed individually in 1 ml sterile water, and dilutions plated on NA amended with appropriates antibiotic markers. Total number of colonies for each strain was recorded.

## RESULTS AND DISCUSSION

**Mutagenesis of *carAB* and *rpoS* Gene**

Whole nucleotides of *carA*, *carB*, and *carAB* genes of SP007s encoding the small subunit A (CPSase A), large subunit B (CPSase B), and CPSaseAB was obtained by deletion mutants using overlap extension PCR. The sequencing of clones obtained from each mutant plasmid (pTok2::Δ*carA*, pTok2::Δ*carB*, and pTok2::Δ*carAB*) was shown to harbor part of different subunits that revealed a high similarlity to those reports of several species in *Pseudomonas* (83-100%) including *P. fluorescens* L111 and *P. fluorescens* L321 (Accession: CP015638.1 and CP015637.1); *P. protegen* pf-5 and *P. protegen* UCT (Accession: CP015639.1 and CP017964.1); *P. aeruginosa* PAO1 and *P. aeruginosa* PA96 (Kwon *et al.*, 1994, Accession: CP007224.1); and *P. putida* E46 and *P. putida* E41 (Accession: CP024086.1 and Accession: CP024085.1). The plasmids carrying different fragment chromosomes of SP007s to establish in strains SP007s*carA*$^-$, SP007s*carB*$^-$, and SP007s*carAB*$^-$ mutants were carried an insertion in their corresponding genes (Figure 1A). The PCR products using internal primer that recognized the fragment sizes of 517 and 1,140 bp encoding a protein by the corresponding gene *carA* and *carB* with theoretical size of 42 kDa and 118 kDa was found in different SP007s strains constructed. The complemented and overexpression strains were constructed with those corresponding fragments as described (Table 1). These strains compared to parental strain were tested for gene function of CPSase biosynthesis that the corrected results were observed as expected (Figure 7).

In addition to *carAB* mutation, strain *rpoS* mutant was constructed in SP007s chromosome using the same overlap extension method (Figure 1). It was used to evaluate the expression of target genes that the result was described later in Figure 1B. The PCR product using internal primer that recognized the fragment size of of 202 bp encoding a protein by the corresponding *rpoS* gene was found in only parental strain.

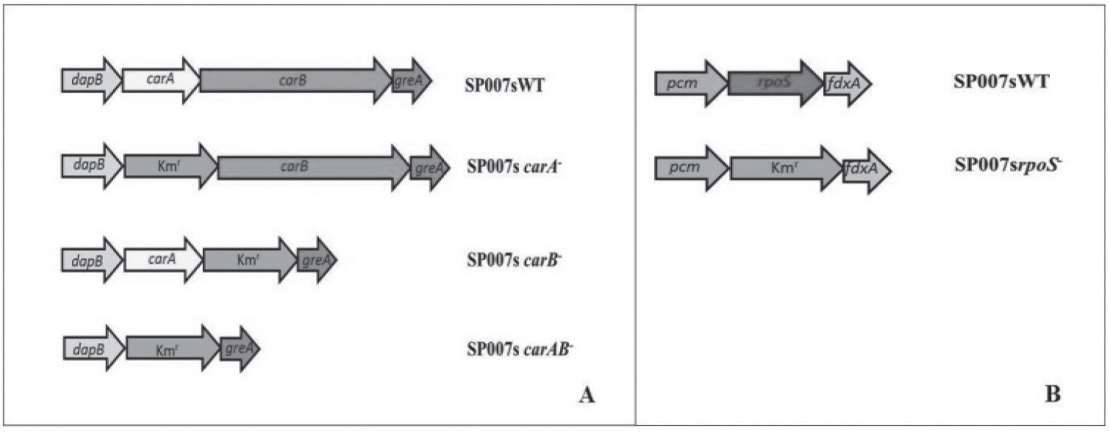


**Figure 1** Physical map of the *carAB* (A) and *rpoS* (B) regions from plasmid and strains *Pseudomonas fluorescens* SP007s used in this study. The positions of target gene and antibiotic gene insertion were shown

**Phenotypic Effects of Mutants**

The *carAB* and *rpoS* were found associated to the successful survival of *P. fluorescens* SP007s such as cell proliferation, motility ability, biofilm formation, EPS production and extracellular protease. As shown in Figure 2, mutants exhibited a reduction of OD and cell populations at all selected times determined, as compared to SP007sWT and complemented strains, meanwhile SP007sOX (*carAB*-overexpression) grew more rapidly than those of 9 strains tested. The *car* gene deletion mutants had significantly impaired growth (indicated by final population size) after culturing for 72 hrs in LB broth suggesting *carAB* was probably involved in SP007s growth that might contribute to decrease control ability of *carAB* mutant. This effect might be due to the *carAB* encoded CPSase the catalyzed ATP dependent in carbamoylphosphate synthesis that involved with pyrimidine and arginine biosynthesis of bacteria (Guy *et al*., 1998; Touratier *et al*., 1999; Newman *et al*., 2008; Tarighi and Taheri, 2011).

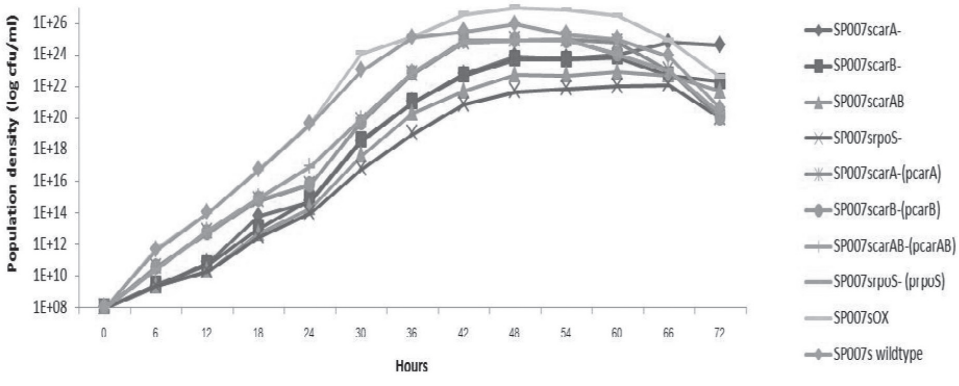


**Figure 2** Mutation of *carA*, *carB*, *carAB*, and *rpoS* reduced growth of *Pseudomonas fluorescens* S P007s in LB broth medium. Complemented strains restored their growth where *carAB* overexpression (SP007sOX) grew more rapidly, as compared to wildtype

As expected, SP007sOX and SP007s*rpoS*$^{-}$ exhibited a higher speed phenotype on tested medium (5.2 cm) after 24 hrs inoculation. As shown in Figure 3A, all *car* genes mutant strains reduced ability to spread in the motility agar compared with SP007sWT. For biofilm formation, SP007sOX and SP007s*rpoS*$^{-}$ were formed visible biofilm in wells after continuous agitation and produced 10% and 15% greater biofilm SP007sWT. On the other hand, all mutants exhibited reduced biofilm. A double mutant (SP007s*carAB*$^{-}$) produced significantly levels of biofilm compared to wildtype and it formed less biofilm than single alone mutants of either SP007s*carA*$^{-}$ or SP007s*carB*$^{-}$ that exhibited a significant reduction in biofilm formation (Figure 4A). Biofilm formation in most gram-negative bacteria was directly enhanced by the QS system, disruption of this signal by CPSase from SP007s then, resulted in reduced biofilm formation (Fuqua *et al.,* 1994). Besides DSF signal was available in almost xanthomonads, other bacteria such as bacilli produced AHL (N-acyl homoserine lactones) for their cell-to-cell communication (Kaplan and Greenberg, 1985). This QS system is then dependent on the production and perception of AHL by two subunits (CPSase A and B) belonging to the *carAB* genes. Mutation in *carAB* was therefore no effect on cell-to-cell communication and the antagonistic bacteria itself had less ability to control the pathogen.

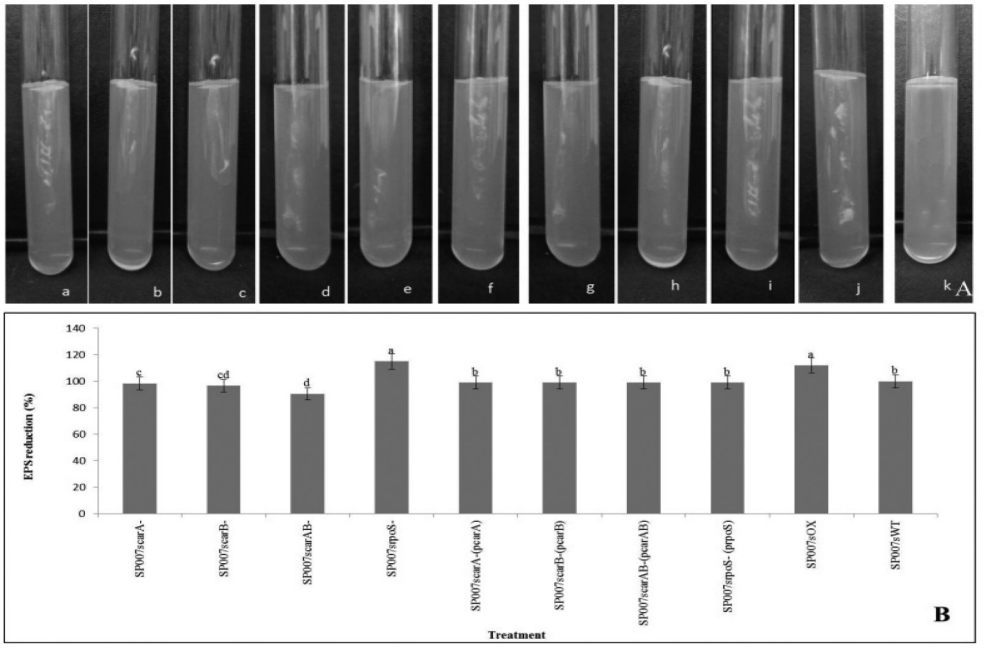


**Figure 3** Effect of *carAB* and *rpoS* genes on *Pseudomonas fluorescens* SP007s motility (A) and extracellular polysaccharide (EPS) production (B). A, motility of SP007s *carA*$^{-}$ (a), SP007s *carB*$^{-}$ (b), and SP007s *carAB*$^{-}$ (c), SP007s*rpoS*$^{-}$ (d), complement strain SP007s*carA*$^{-}$ (p*carA*) (e), SP007s*carB*$^{-}$ (p*carB*) (f), SP007s*carAB*$^{-}$ (p*carAB*) (g), SP007s*rpoS*$^{-}$ (p*rpoS*) (h), and SP007sOX (i) compared with wildtype (j) and dd$H_2O$ (k) after stab-inoculated on motility medium and incubated at 28°C for 24 hrs. The distance of all migration from medium sample to the bottom of test tube was conducted with visualized measurement. B, *carAB* and *rpoS* mutants affected EPS production in NYGB medium. Different letters indicate significant differences between treatments as determined by DMRT ($P \leq 0.05$)

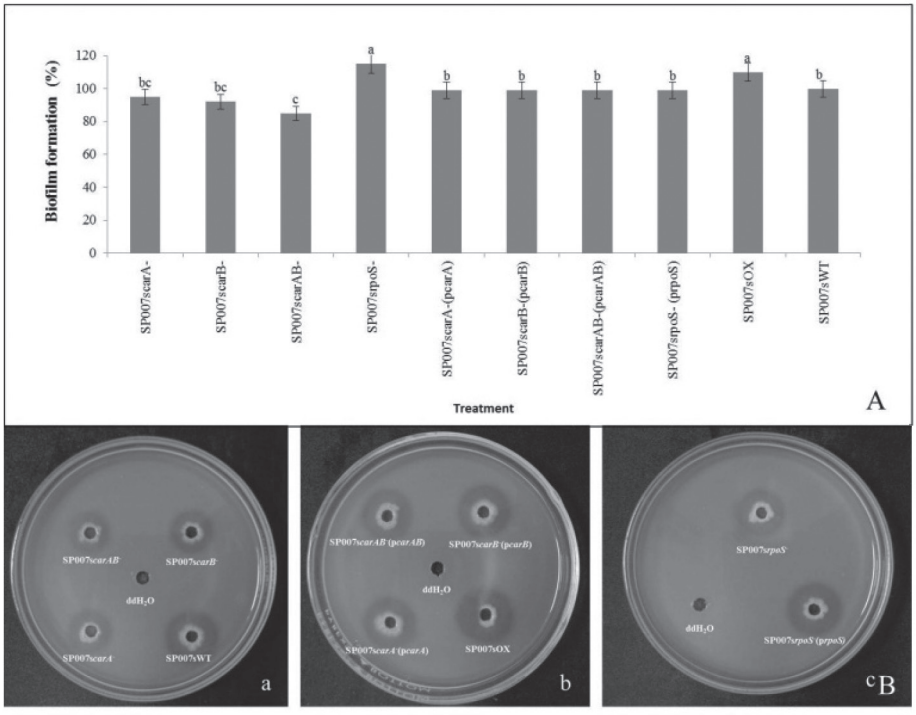


**Figure 4** Phenotypic effects of the *car* and *rpoS* genes in *Pseudomonas fluorescens* SP007s to biofilm and protease productions. A, biofilm formation of wildtype, mutants, and complemented strains cultured in LB within 96-well plates assessed determined by spectrophotometer after staining with crystal violet and normalized to cell density measured at 600 nm. Different letters indicate significant differences as determined by DMRT ($P \leq 0.05$). B, culture filtrates were placed in well on test medium to protease activity assessed. Mutation of *carAB* and *rpoS* affected to protease secretion that mutants produced smaller-, where SP007sOX displayed larger-clear halos compared with wildtype

The SP007sOX and and SP007s*rpoS*$^-$ produced EPS 12% and 15% greater than SP007sWT while *car* gene mutants produced EPS less than parental strain SP007s. Mutants SP007s*carA*$^-$, SP007s*carB*$^-$ and SP007s*carAB*$^-$ exhibited the 1.6, 3.4 and 9.5% respectively were reduction in EPS as compared with SP007sWT (Figure 3B). These evidences were similar to *carAB* mutant strain of *Halomonas eurihalina* that also deficient in EPS production (Llamas *et al.*, 2003). This might be expected that *carAB* affected deficiency in the UDP-sugar pool. This sugar is essential to utilize in the synthesis not only of EPS but also of lipopolysaccharides and the glycosylation of lipids and fatty acids (Newman *et al.*, 2008). Ability of *carAB* gene from *Pseudomonas* spp. strain G encoded CPSase that was required also for rapid degradation of DSF. Its *carAB* mutants in *Pseudomonas* spp. strain G involved deficient in EPS production (Newman *et al.*, 2008) was also reported. In grape used to coinoculation *Pseudomonas* spp. strain G and *Xylella fastidiosa* was found disruption of DSF-mediated signaling suppressed involved reduced plant pathogen population (Zhang, 2003). Disruption of QS therefore, could be recognized as one of mechanisms by bacterial antagonists in suppressing pathogen activity.

The result of mutants reduced levels of protease activity whereas the complement strains showed protease production similar to the SP007sWT indicating *carAB* might activate the catalyzed hydrolytic reactions resulting in facilitated protease synthesis (Blanco *et al.*, 2016). However, genetic route of *carAB* involved in these gene regulatory system was unrecovered in this study.

In addition, all of *car* gene mutants reduced levels of protease activity whereas the complemented strains showed protease production similar to the SP007sWT. Among mutant strains, double mutant (SP007s *carAB*$^-$) exhibited the greater activity of protease than single mutants SP007s *carA*$^-$ and SP007s *carB*$^-$ alone. A clear halos surrounding the wells of bacterial inoculate that indicated a positive for protease activity exhibited 0.3, 0.3, 0.3, 0.2, 0.5 and 0.7 mm in diameter was regulated by SP007s*rpoS*$^-$ SP007s *carA*$^-$, SP007s *carB*$^-$, and SP007s *carAB*$^-$, SP007sWT and SP007sOX, respectively (Figure 4B).

More importantly, strains SP007sOX and SP007s*rpoS*$^-$ had highest inhibitory effect against Xag 12−2 (1.0 cm of halo) then, SP007sWT (0.9 cm), single mutant of either SP007s *carA*$^-$ and SP007s *carB*$^-$ strains significantly reduced in ability to suppress the pathogen compared to wildtype by agar plate bioassay with 0.7 cm (SP007s*carA*$^-$), 0.6 cm (SP007s*carB*$^-$) and 0.5 cm (SP007s*carAB*$^-$), respectively (Figure 5). Previous studies demonstrated that *P. fluorescens* SP007s have had ability to reduce development of multiple diseases in different economic crops including bacterial pustule of soybean (Prathuangwong, 2016; Prathuangwong *et al*., 2009; 2012). They also included the establishment of secondary metabolites (such as phenazine, herbicolin, pyoluteorin, pyrrolnitrin, and 2,4-DAPG) produced by SP007s (Kladsuwan *et al*., 2013a; 2013b). Thus, SP007s*carAB*$^-$ was significantly reduced an ability to suppress the Xag colonization suggesting the effects of no biosynthesis CPSase product and other abolished phenotypes.

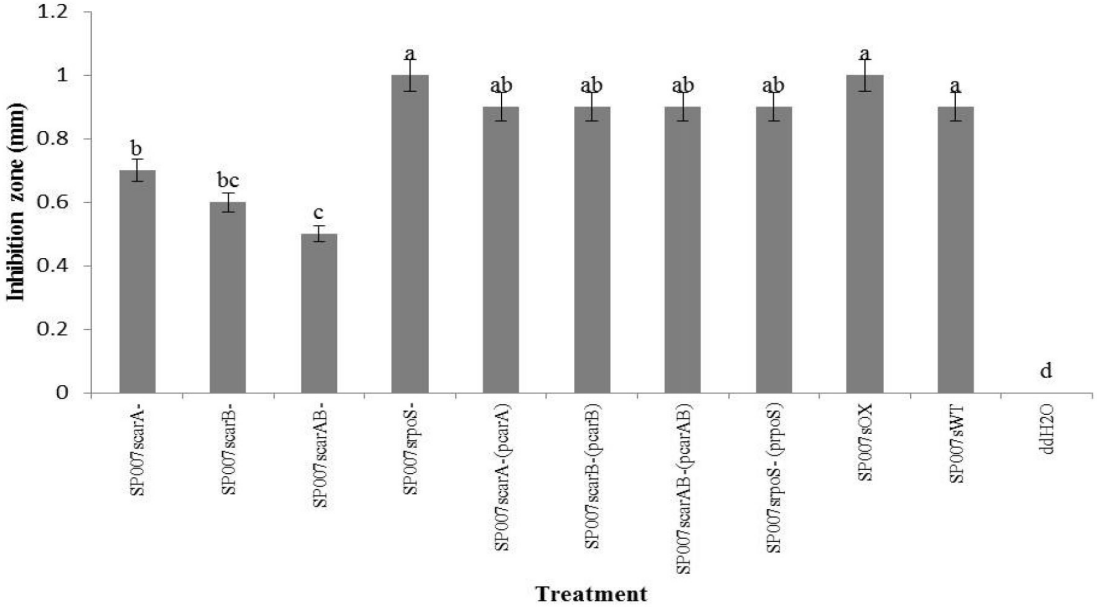


**Figure 5** Inhibition effect of *Xanthomonas axonopodis* pv. *glycines* 12-2 by *Pseudomonas fluorescens* SP007s mutants, SP007sOX and complement strains compared with wildtype. Filter discs saturated with each SP007s strain suspension was placed on NA plate containing Xag 12-2 and inhibitory was measured after 48 hrs incubation. Different letters indicate significant differences as determine by DMRT ($P \leq 0.05$)

**The CPSase Purification**

According to several bacterial antagonists, CPSase activity is contained in a complex encoded by two genes, *carA* and *carB* (Ahuja *et al*., 2001; Kilstrup *et al*., 1988; Kwon *et al*., 1994; Piette *et al*., 1984). A particular enzyme named CPSase that catalyzed ATP dependent the synthesis of carbamoylphosphate synthetase from glutamine and ammonia, a precursor for pyrimidine and arginine biosynthesis encoded by *carAB* in various bacterial antagonists can rapidly degrade DSF produced by pathogenic *Xanthomonas* species (Newman *et al*., 2008; Tarighi and Taheri, 2011; Guy *et al*., 1998). The small subunit of this enzyme complex, a glutamine amidotransferase encoded by *carA* where *carB* encodes and catalyzes the large subunit, synthesis of carbamoylphosphate from ammonia, bicarbonate, and ATP (Kwon *et al*., 1994; Lu *et al*., 1997). Several bacterial antagonists have been identified for cell-cell signal degradation via specific enzyme activities (Dong and Zhang, 2005; Gonzalez and Keshavan, 2006) such as genera *Bacillus*, *Paenibacillus*, *Microbacterium*, *Staphylococcus*, and *Pseudomonas* etc.

All SP007sWT and mutant strains tested showed clear CPSase production correlated to *carA*, *carB* and *carAB* expression which were detected and purified by TLC, SDS-PAGE, and HPLC. These analyses revealed that small and large subunit of CPSase obtained from either single or double mutants, or SP007sWT were identical with purified CPSase (catalog number MBS966352) that was used for standard comparison as the earlier report (Corvi *et al*., 2001; Mccudden *et al*., 1996; Robino *et al*., 1987). A single band of light molecular weight of 42 kDa was detected in SP007s*carB*$^{-}$, where a single band associated with heavy molecular weight of 118 kDa was obtain in SP007s*carA*$^{-}$. SP007s*carAB*$^{-}$ strain was incapable of synthesizing any detected amount of small and large subunit. Complementation was restored to parental strain whereas strain SP007sOX showed a greater concentration of CPSase than in the SP007sWT strain. This suggesting that *carB* gene might affect *carA* transcript although it consistently served as an mRNA for corresponding-small subunit of CPSase. The *carB* complementation (p*carB* in lane 6) resulted in high expression of the small subunit equal to that of SP007sWT confirming *carB* deletion affected expression of the small subunit. Result of CPSase biosynthesis obtained from SDS-PAGE was consistent with the TLC analyses indicating that the culture filtrate extracts of different SP007s strains contained either small or large subunit, or both of CPSase except the strain SP007s*carAB*$^{-}$ (Figure 6 and Figure 7).

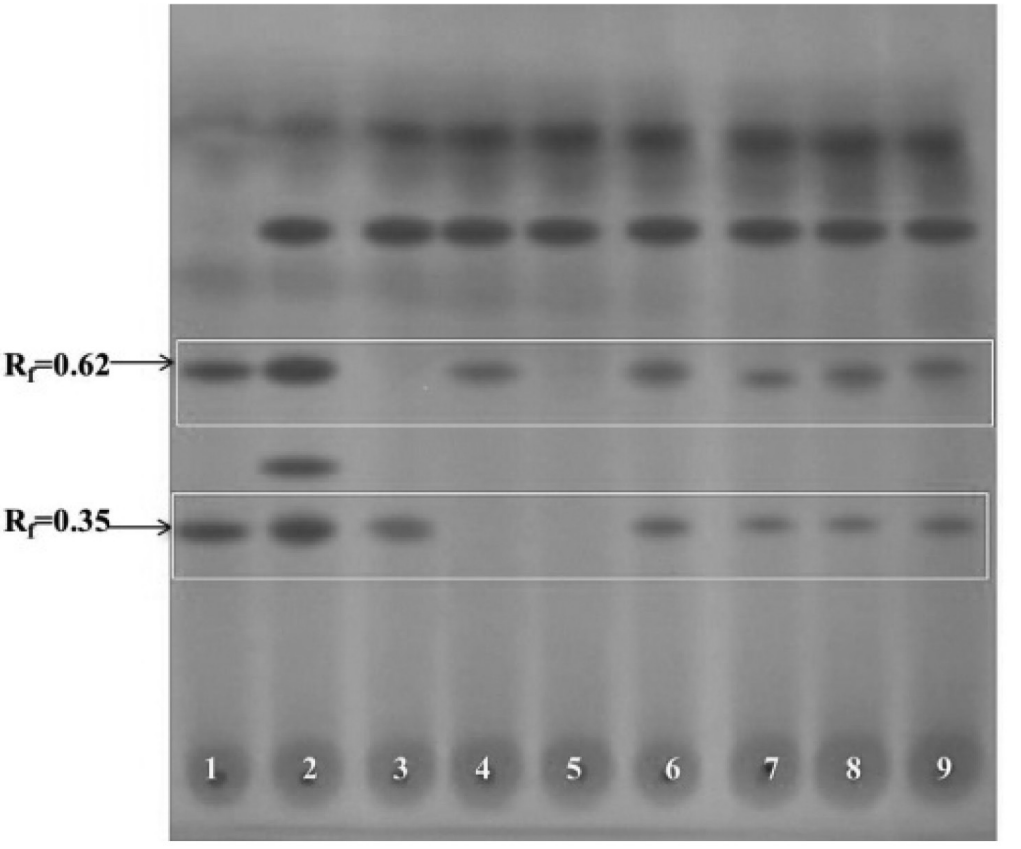


**Figure 6** Visualized-large subunit ($R_f$ = 0.35) and small subunit ($R_f$ = 0.62) of carbamoyl phosphate synthetase (CPSase) of *Pseudomonas fluorescens* SP007s, mutants, complemented strains separated by TLC. Lanes 1 = marker (catalog number MBS966352), 2 = SP007sOX, 3 = SP007s*carA*$^-$, 4 = SP007*scarB*$^-$, 5= SP007s*carAB*$^-$, 6 = SP007s*carA*$^-$ (p*carA*), 7 = SP007s*carB*$^-$ (p*carB*), 8 = SP007s*carAB*$^-$ (p*carAB*), 9 = and wildtype

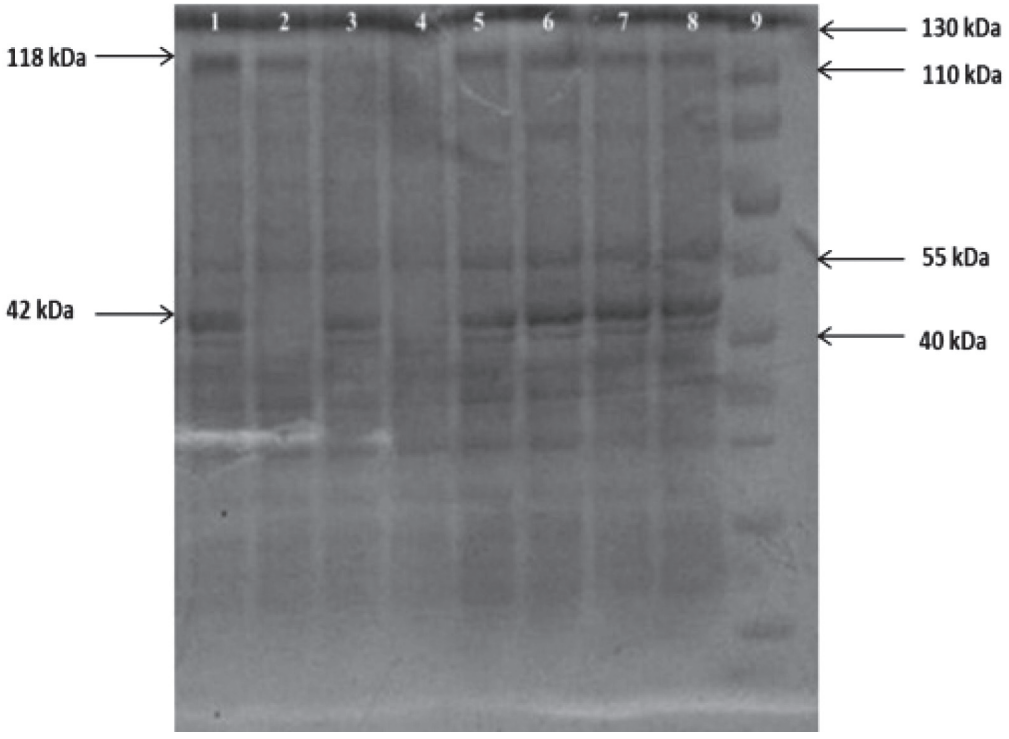


**Figure 7** Secreted CPSase detected by SDS-PAGE that the load proteins were extracted from supernatants of *Pseudomonas fluorescens* SP007s, mutants, and complemented strains. The size of two subunits, 118 kDa and 42 kDa present in the left margin was revealed from their migration relative to standard of known molecular weight; lanes 1 = SP007sOX, 2 = SP007s*carA*$^{-}$, 3 = SP007*scarB*$^{-}$, 4 = SP007s*carAB*$^{-}$, 5 = SP007s*carA*$^{-}$ (p*carA*), 6 = SP007s*carB*$^{-}$ (p*carB*), 7 = SP007s*carAB*$^{-}$ (p*carAB*), 8 = wildtype and 9 = marker

The CPsase from each SP007s strain was determined by HPLC which the standard marker (catalog number MBS966352) was used in the comparison. The results obtained clearly distinguished small subunit from the large subunit of CPSase regarding to peak area and the retention time (RT). The two peaks shown in Figure 8, demonstrated the area of small and large subunit obtained from SP007sWT that had the RT of about 2.0 and 2.6 min, respectively. The combined CPSase fractions yielding with HPLC indicated that the small and large subunit for about 4.67 and 16.31 μmol/min$^{-1}$mg protein$^{-1}$ of the soluble cell protein collected from 1L of each strain SP007s culture supernatant, respectively. As expected, no CPSase full length, CPSase subunit A, or CPSase subunit B were detected by HPLC in extracts from strains SP007s*carAB*$^{-}$, SP007s*carA*$^{-}$ or SP007s*carB*$^{-}$, respectively, whereas the complemented strains exhibited wildtype CPSase production levels (data not shown). Detection of CPSase produced by each SP007s strain with different analyses indicated the presence of homologous compounds belonging to the same class of enzyme and confirmed that the methods applied efficiently separated CPSase from the bacterial cells tested.

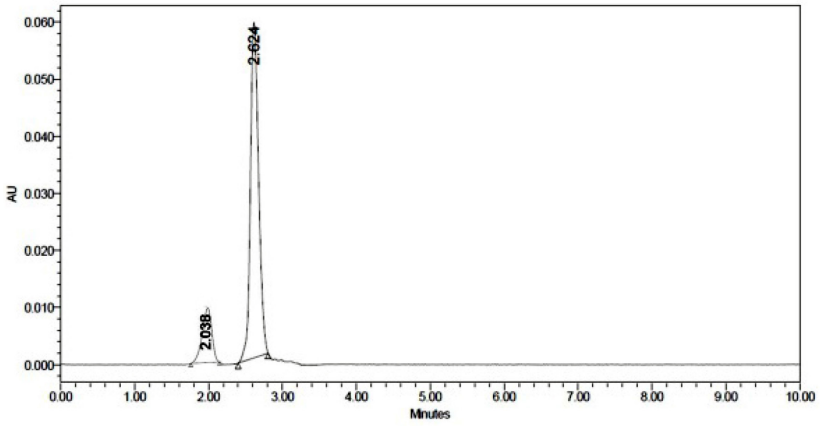


**Figure 8** Comparison of the peak area and RT of CPSase obtained from *Pseudomonas fluorescens* SP007s using HPLC. The RT of 2.0 and 2.6 was observed for small and large subunit, respectively

To verify the CPSase activity measured was a function of the large or small subunit alone, expression of *car* genes was carried out under stationary phase of SP007sWT and mutants grown in LB using qRT-PCR analysis. The expression of individual gene (*carA* or *carB*) was in reasonable accordance with exogenous secreted enzyme obtained from CPSase measurement and transcript levels that responding to its subunit A or B. Expression of only SP007s*carA*$^-$ in the SP007s*carB*$^-$ strain was observed which was consistent with the result of CPSase subunit A yield in SP007s*carB*$^-$ supernatant. *CarB* clearly functioned alone in SP007s*carA*$^-$ whereas strain SP007s*carAB*$^-$ lost the expression of *car* genes which was related to incapable of synthesizing any detectable subunit A or B. A small increase in expression of *carAB* genes in SP007sOX was found compared to SP007sWT (Figure 9).

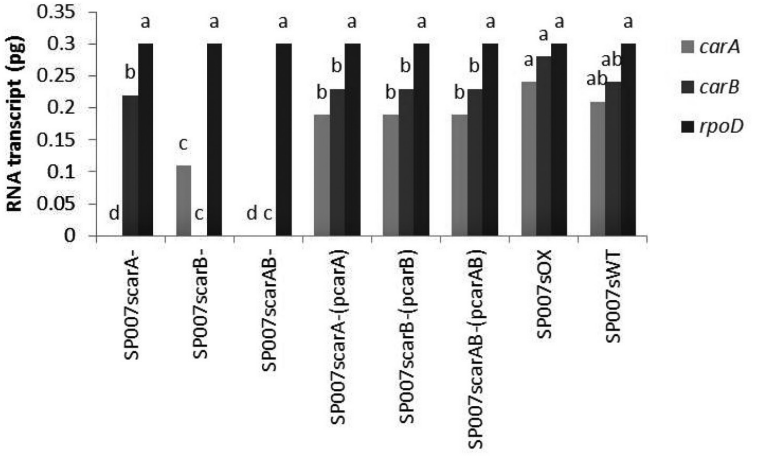


**Figure 9** Expression of *carAB* of *Pseudomonas fluorescens* SP007s, mutants, and complemented strains in basal salts medium

**Assay of Quorum Sensing Disruption**

DSF is a fatty acid signal molecule involved in regulation of virulence in *X. campestris* pv. *campestris*, *X. oryzae* pv. *oryzae, X. axonopodis* pv. *citri* and *X. axonopodis* pv. *glycines* known to have the Rpf system for production and detection of DSF (Tang *et al.,* 1991; Barber *et al.,* 1997; Chatterjee and Sonti, 2002; Thowthampitak *et al.,* 2008). *X. axonopodis* pv. *glycines* strain KU-P-34070 produced DSF related quorum sensing molecule produced by other *Xanthomonas* spp. Mutations in *rpfF* mutants of Xag strain KU-P-34070 were defective in the production of DSF and reduce virulence on soybean plants (Thowthampitak *et al.,* 2008). This *rpfF* mutant strain KU-P-34070 exhibited also phenotypic effects on other survival traits similar to SP007s *carAB* mutant such as biosynthesis of EPS.

Strains SP007sWT, mutants and CPSase overexpression including purified CPSase from culture were tested for their ability to degrade DSF biosynthesis of pathogenic *X. axonopodis* pv. *glycines* 12−2. The complete darkness areas (a large zone of DSF degradation) in Figure 10G exhibited a strong degradation of *X. axonopodis* pv. *glycines* 12−2 bioluminescence by strain SP007sOX that produced higher level of CPSase than its parental strain. The extracts were (separated by either TLC, SDS-PAGE or HPLC function) similar to that of strain SP007sOX whereas SP007sWT showed lower degree at degradation (Figure 10H). Three-mutant strains exhibited different degrees of DSF degradation that SP007s*carAB*$^{-}$ strain showed large zone of brightly fluorescence agar plate (Figure 10C) compared to SP007s*carA*$^{-}$ (Figure 10A) and SP007s*carB*$^{-}$ (Figure 10B). This suggesting that CPSase produced by SP007s viable cell (coculture) affected only DSF biosynthesis but not reduced cell growth of *X. axonopodis* pv. *glycines* 12−2. The effectiveness of CPSase subunit A and subunit B separately was similar capacity on DSF degradation. The large subunit B in strains SP007s*carA*$^{-}$ activity (Figure 10A) however seemed to reduce higher level of DSF production compared with that of activity in SP007s*carB*$^{-}$ of small subunit A synthesis (Figure 10B). This evidence was agreement with the amount of more subunit B (thick spot on gel electrophoresis) obtained from SDS-PAGE extracts (Figure 7 lane 8) and HPLC fraction. The complementation strains SP007s*carA*$^{-}$ (p*carA*), SP007s*carB*$^{-}$ (p*carB*), and SP007s*carAB*$^{-}$ (p*carAB*) exhibited wildtype levels of DSF degradation in this study (Figure 10D, 10E and 10F).

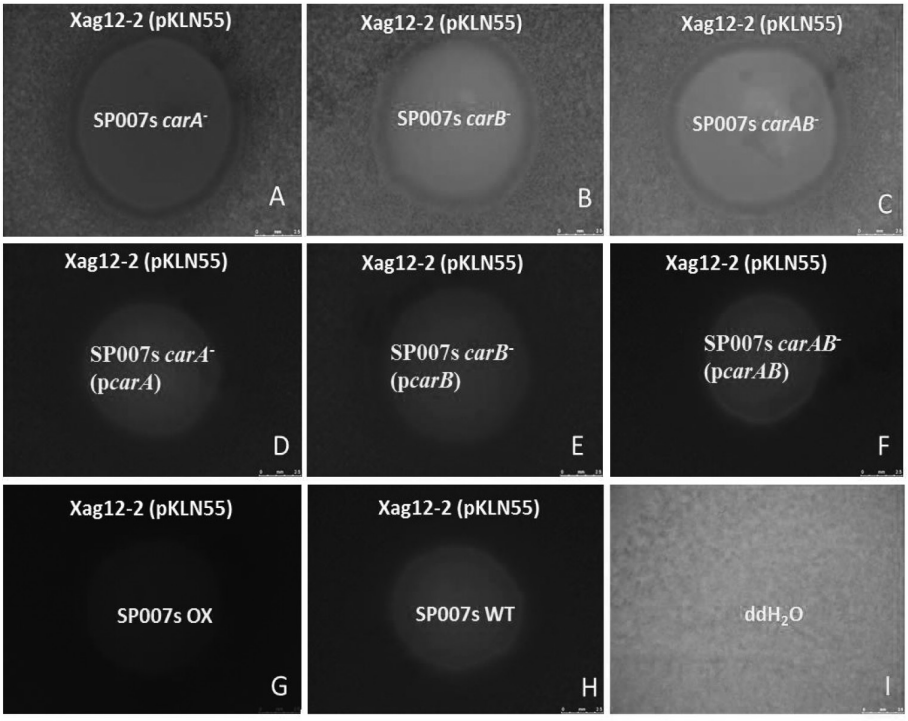


**Figure 10** Degradation of diffusible signal factor (DSF) in *Xanthomonas axonopodis* pv. *glycines* 12−2(pKLN55) by *Pseudomonas fluorescens* SP007s (CPSase), which a green fluorescent protein reporter gene (*gfp*) fused to DSF-inducible promoter region of *engXCA*. Xag 12−2 airbrush inoculation on NA as basal layer and then drop each suspensions of SP007s strain including SP007s*carA*$^-$ (A), SP007*scarB*$^-$(B), SP007s*carAB*$^-$(C), SP007s*carA*$^-$ (p*carA*) (D), SP007s*carB*$^-$ (p*carB*) (E), SP007s*carAB*$^-$ (p*carAB*) (F), SP007sOX (G) and wildtype (H) onto the center (SP007s) of Xag agar plate. Presence of Xag growth/ DSF production with green fluorescent was examined by *Leica* M205 fluorescence stereomicroscope after 24 hrs incubation. Xag growth with $dH_2O$ (no CPSase) expresses high capacity of DSF secretion brightly green fluorescence (I) compared to darkness zone of a strong degradation of DSF by SP007sOX (G)

**Analysis of Gene Expression**

The experiment detects the effect of carbamoyl phosphate synthetase (*carA* and *carB*), pyochelin siderophores (*pch*), phenazine (*phzE*), 2,4-DAPG (*phl*), pyoluteorin (*plt*), and sigma factor S (*rpoS*) was carried out under stationary phase of SP007sWT and mutants grown in M9 minimal medium using qRT-PCR analysis. In SP007s*carAB*$^{-}$ found that *carAB* gene have effect to interfered with 2,4-DAPG, pyoluteorin, phenazine, pyochelin, and sigma factor S with 0.14, 0.18, 0.13, 0.16 and 0.18 pg, respectively whereas SP007sWT these gene expression with 0.17, 0.21, 0.16, 0.21, 0.18 and 0.28 pg, respectively (Figure 11A). Mutant SP007s*carAB*$^{-}$ affected multiple gene expression encoding extracellular products that included secondary metabolites associated with biocontrol traits. Mutations in *carAB* caused a decreased expression of *phl*, *plt*, *phzE*, *pch* (corresponding to 2,4-DAPG, pyoluteorin, phenazine, and pyochelin siderophore biosynthesis), and *rpoS*; whereas *rpoS* mutant mediated decreased production activity a of *carAB* expression suggesting these gene regulation required a functional *carAB* genes. The overproduced 2,4-DAPG and pyoluteorin in SP007s*rpoS*$^{-}$ were found that gene maybe due to *rpoS* required a transcription of a gene encoding a protein that functions as a repressor of pyoluteorin and 2,4-DAPG biosynthesis in *P. fluorescens*. Alternatively, sigma S may serve as a negative regulator by competing for association with the RNA polymerase core with another sigma factor S required for production of 2,4-DAPG and pyoluteorin (Pfender *et al*., 1993; Sarniguet *et al*., 1995).

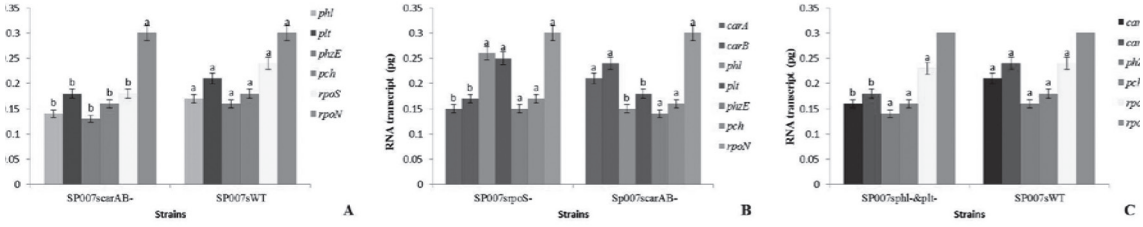


**Figure 11** Expression analysis of *carA*, *carB*, *phl*, *plt*, *phzE*, *pch*, *rpoS* and *rpoD* gene in SP007s*carAB*$^{-}$, SP007s*rpoS*$^{-}$, SP007s*phl*$^{-}$*& plt*$^{-}$ and SP007s*phzE*- compared with wildtype culture grown in M9 minimal medium

**Determination of Antimicrobial Compound**

All strains of SP007s tested produced 2,4-DAPG, pyoluteorin and phenazine production with standard enzyme by TLC tested. Antibiotic identity was established by the appearance of a specific band were visualized under UV light with $R_f$ values and 0.35, 0.5 and 0.5, respectively. The 2,4-DAPG, pyoluteorin, and phenazine antibiotic produced by strain SP007s has been reported to responsible for different portion of the biocontrol of soybean bacterial pustule showed reduction in disease suppression against *X. axonopodis* pv. *glycines*.

Siderophore production were detected from *P. fluorescens* SP007s. Siderophore is an iron chelating compound that secreted by overexpression (SP007sOX), mutants (SP007s*carA*$^{-}$, SP007*scarB*$^{-}$, SP007s*carAB*$^{-}$, SP007s*phl*$^{-}$*&plt*$^{-}$ and SP007s*rpoS*$^{-}$) and complemented strains SP007s*carA*$^{-}$ (p*carA*), SP007s*carB*$^{-}$ (p*carB*) and SP007s*carAB*$^{-}$ (p*carAB*) compared with parental strain (SP007sWT) that demonstrated their ability produced siderophore on CAS-PSA medium, were detected by orange halo zone surrounding bacterial growth exhibited 1.5, 1.0, 1.0, 0.8, 1.2, 0.8, 1.2, 1.2, 1.3,1.3 and 1.3 cm, respectively. The result indicated that the *carAB* gene of *P. fluorescens* SP007s had been effect on siderophore production (Figure 12).

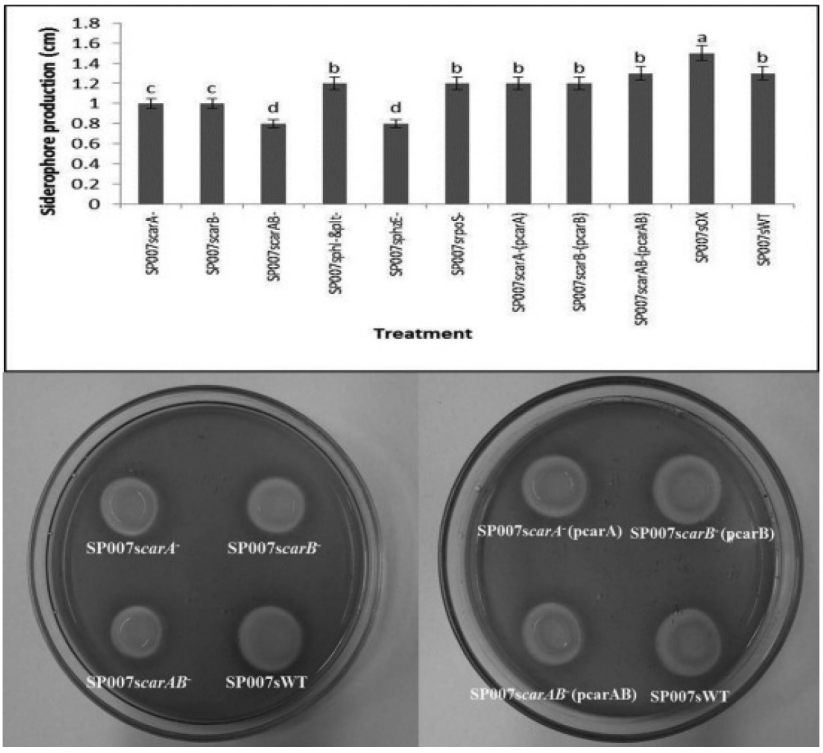


**Figure 12** Phenotypic effects of the *car* and *rpoS* genes in *Pseudomonas fluorescens* SP007s were placed on CAS-PSA medium to assess siderophore production. Mutation of *carAB* affects siderophore production that mutants produced a smaller-, where SP007sOX display a larger-orange halos compared with wildtype

Determination of siderophore production, the result showed that overexpression produced siderophore better than mutants, complemented strains and wildtype. Strain SP007s could compete with plant pathogens in various needs such as food, air and space with high amount of siderophore production (Prathuangwong *et al*., 2009). Siderophore is a specific catching of the ion radicles in soils. In competition among biocontrol agent and pathogen, biocontrol agent can reduce the amount of nutrients which is essential for the growth of the pathogen because of the biocontrol agent is effective in the use of food (iron uptake) or has the ability to grow very rapidly (Chaiharn *et al*., 2009).

Antimicrobial activity of *phl* and *plt* mutant compared with parental strain (saturated filter paper) against the pathogenic *X. axonopodis* pv. *glycines* 12−2 (inoculated NA plate) evaluated with measuring the halos around paper disc contained each bacterial suspension. Strains SP007sWT had highest inhibitory effect against *X. axonopodis* pv. *glycines* 12−2 (0.9 cm of halo) then, SP007s*plt*$^-$ (0.8 cm), SP007s*phl*$^-$ (0.7 cm), and SP007s*phl*$^-$*&plt*$^-$ (0.5 cm) was significantly reduced in ability to suppress the pathogen compared to wildtype by agar plate bioassay suggesting *phl*$^-$ *& plt*$^-$ in SP007s influenced the cell proliferation of strain 12−2 (Figure 13).

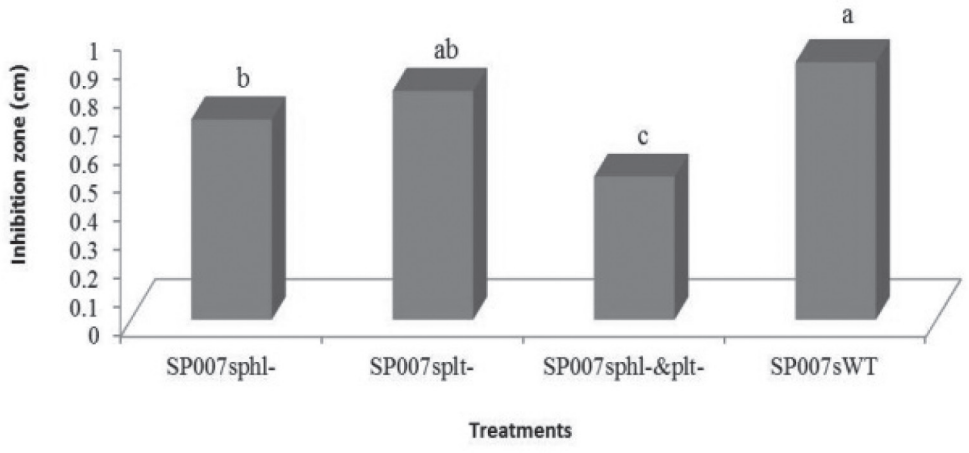


**Figure 13** Inhibition effect of Xag 12−2 by SP007s*phl*$^-$, SP007s*plt*$^-$, and SP007s*phl*$^-$ & *plt*$^-$ compared with SP007sWT. Filter discs saturated with each SP007s strain suspension was placed on NA plate containing Xag 12−2 and inhibitory was measured after 48 hrs incubation. Bars point indicated significant differences as determined by DMRT (P ≤ 0.05)

**The Role of Gene Linkage**

In SP007s*rpoS*$^-$ found that *rpoS* gene have effect to interfered with carbamoyl phosphate synthetase (*carA* and *carB*), 2,4-DAPG, pyoluteorin, phenazine, pyochelin, and house keeping gene (*rpoD*) with 0.15, 0.17, 0.26, 0.25, 0.15, 0.17 and 0.30 pg, respectively whereas SP007sWT these gene expression with 0.21, 0.24, 0.17, 0.21, 0.16, 0.18 and 0.30 pg, respectively (Figure 11B). Similarity, *rpoS* mutant of Pf-5 is altered in secondary metabolite production, overproducing pyoluteorin and 2,4-DAPG, and lacking production of pyrrolnitrin (Pfender *et al*., 1993; Sarniguet *et al*., 1995). Instead, the *rpoS* gene maybe required for transcription of a gene encoding a protein that functions as a repressor of pyoluteorin and 2,4-DAPG biosynthesis in *P. fluorescens*. Alternatively, sigma S may serve as a negative regulator by competing for association with the RNA polymerase core with another sigma factor S required for production of 2,4-DAPG and pyoluteorin. Production of secondary metabolites and exoenzymes by *P. fluorescens* is likely to be controlled by a complex, multicomponent regulatory cascade in which *rpoS*, *apdA*, and *gacA* are but three of many regulatory loci (Sarniguet *et al*., 1995).

In SP007s*phl*$^-$ & *plt*$^-$ found that *phl* and *plt* gene have effect to interfere with carbamoyl phosphate synthetase (*carA* and *carB*), phenazine, pyochelin, sigma factor S, and house keeping gene (*rpoD*) with 0.16, 0.18, 0.14, 0.16, 0.23 and 0.30 pg, respectively whereas SP007sWT these gene expression with 0.21, 0.24, 0.16, 0.18, 0.24 and 0.30 pg, respectively (Figure 11C).

**Coinoculation on Soybean Plant**

To evaluate the potential of CPSase produced by *P. fluorescens* SP007s in degradation of DSF from Xag 12−2 (pKLN55), caused soybean bacterial pustule that would link to the suppression of disease development, coculture between different strains of SP007s (6 hrs earlier) and Xag 12−2 (pKLN55) was spray-inoculated onto foliage at 3-week old soybean cv. SJ5 conducting under greenhouse experiment. The evidence indicated the potential of strain CPSase-producing *P. fluorescens* SP007s linking to biocontrol mechanism that

could protect plants from Xag 12−2 (pKLN55) infection, although disease had been earlier developed. When SP007sOX was inoculated before Xag 12−2 (pKLN55) the greatest disease reduction of 85% was observed, as compared to Xag 12−2 (pKLN55) -alone inoculation. In CPSase extract, SP007sWT, SP007s*rpoS*$^{-}$, SP007s*carA*$^{-}$, SP007s*carB*$^{-}$, SP007s*carAB*$^{-}$, and SP007s*phl*$^{-}$*&plt*$^{-}$ were effective to reduce disease development with 79, 76, 74, 70, 68 and 51% respectively (Figure 14 and Figure 15). The SP007sOX strain that produced higher abundance of CPSase followed by the purified CPSase extracts gave a better disease reduction as compared with its parental strain suggesting that efficacy of disease control depended on the amount or concentration of CPSase levels which was in agreement with the results obtained from experiments of DSF disruption above.

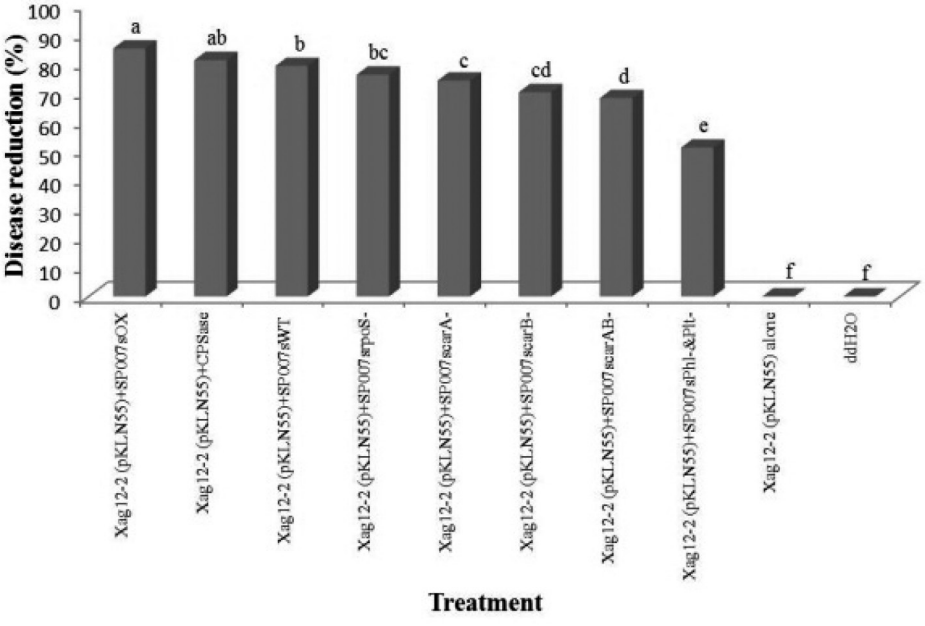


**Figure 14** Percentage of pustule disease reduction with purified CPSase, *Pseudomonas fluorescens* SP007s and mutants primed 6 hrs before Xag-DSF biosensor inoculation on soybean plants

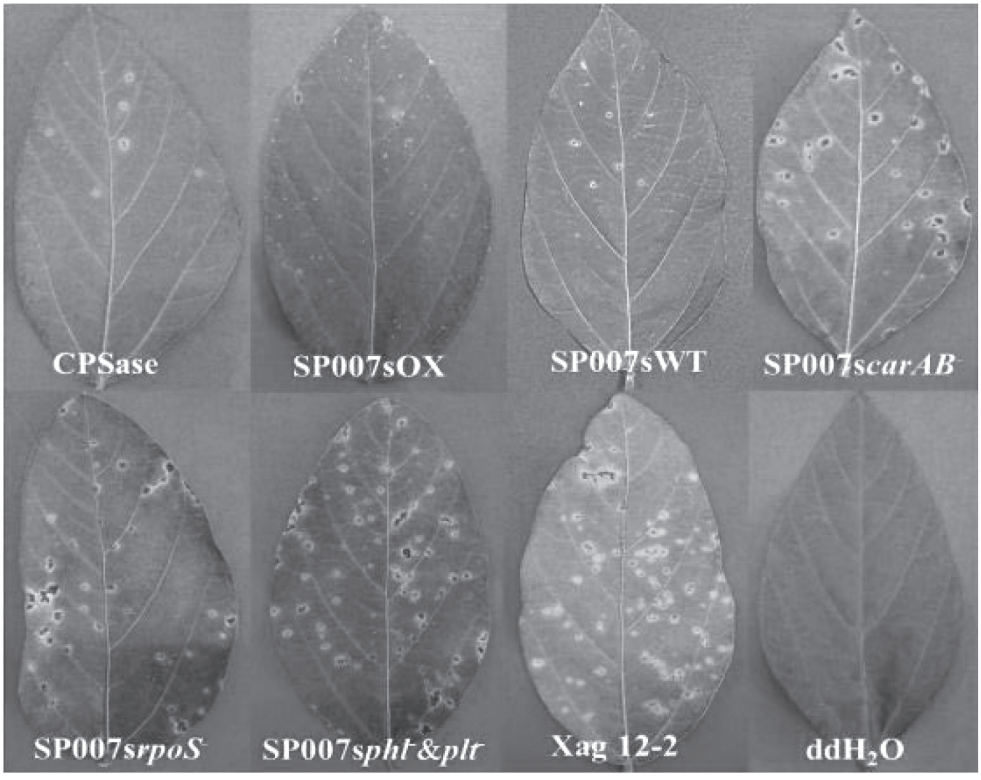


**Figure 15** Disease reduction with purified CPSase, *Pseudomonas fluorescens* SP007s and mutants primed 6 h before Xag-DSF biosensor inoculation on soybean plants

In this study, inoculated with strains SP007s*carAB*- suggesting that other traits might be involved in the control mechanism by *P. fluorescens* SP007s. To verify the traits related to biocontrol of soybean bacterial pustule, two-gene deletion of strain SP007s*phl*- &*plt*- that lost in ability to produce 2,4-DAPG antibiotic and pyoluteorin antibiotics were used to coinoculated with Xag 12−2 (pKLN55). This coinoculation had less efficacy in reducing disease severity as compared with all other strains tested confirming that effective reduction of pustule disease by *P. fluorescens* SP007s might involve various control mechanisms at the same time and that CPSase regulatory system might also a link to biosynthesis of other secondary metabolite.

When inoculated with different strains of SP007s (6 h earlier) before Xag 12−2 (pKLN55) onto foliage at 3-week old soybean cv. SJ5 conducting under greenhouse experiment. Population density of Xag 12-2 (pKLN55)-alone with the greatest Xag population density ($2.27 \times 10^{12}$ cfu/disc) whereas SP007s*phl*-&*plt*-, SP007s*carAB*-, ,SP007s*carB*-, SP007s*rpoS*-, SP007s*carA*-, SP007sWT and CPSase extract were inoculated before Xag 12-2 (pKLN55) have effect Xag population density with $2.16 \times 10^{11}$, $9.36 \times 10^{10}$, $5.38 \times 10^{10}$, $2.01 \times 10^{10}$, $1.02 \times 10^{10}$, $8.67 \times 10^{9}$ and $4.02 \times 10^{9}$ cfu/disc respectively (Figure 16A and Figure 16C).

In the coinoculated treatment, sprayed SP007sOX (6 hrs ealier) before Xag 12−2

(pKLN55) found that the greatest SP007s population density ($9.82 \times 10^{16}$ cfu/disc) whereas SP007s*carA*$^-$, SP007s*carB*$^-$, SP007s*carAB*$^-$, SP007s*phl*$^-$&*plt*$^-$, SP007s*rpoS*- was inoculated before Xag 12−2 (pKLN55) lower than SP007sWT with $7.45 \times 10^{12}$, $6.21 \times 10^{12}$, $9.76 \times 10^{12}$, $7.56 \times 10^{13}$, $1.08 \times 10^{12}$, and $7.64 \times 10^{15}$ cfu/disc respectively (Figure 16B and Figure 16C). According to ability of *carAB* gene from *Pseudomonas* spp. strain G encodes *carAB* that is required for rapid degradation of DSF. It is interesting that *carAB* mutants in *Pseudomonas* spp. strain G involved deficient in EPS production (Newman *et al*., 2008). In grape used to coinoculation *Pseudomonas* spp. strain G and *Xylella fastidiosa* was found disruption of DSF-mediated signaling suppressed involved reduce plant pathogen population (Zhang, 2003). Quorum quenching therefore, could be recognized as one of mechanisms by bacterial antagonists in suppressing pathogen activity.

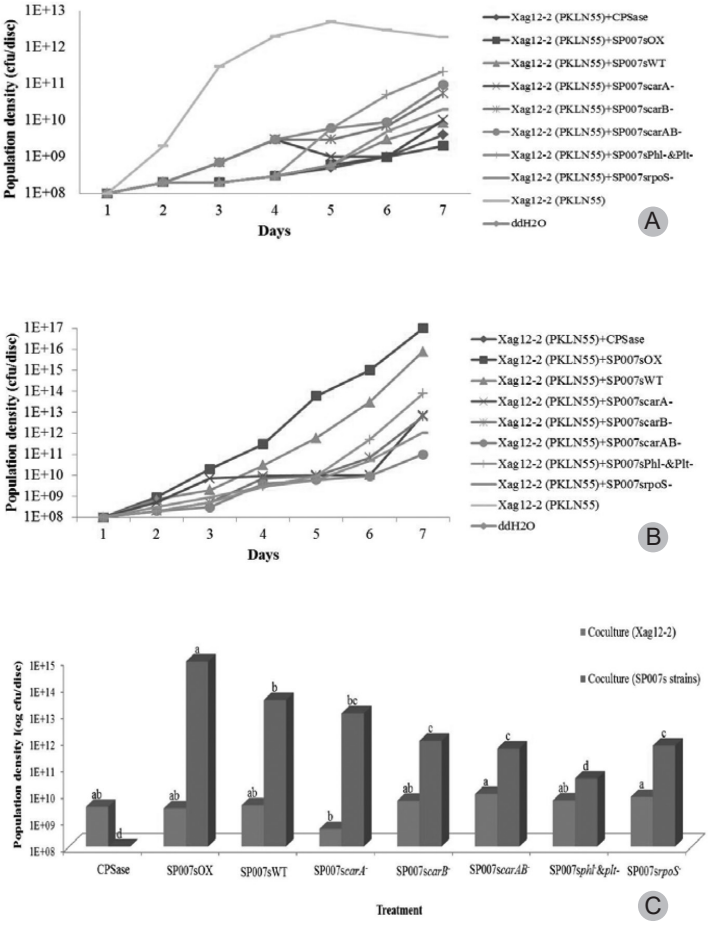


**Figure 16** Survival of different SP007s strains and Xag 12−2 in coinoculation on soybean leaves. Effect of SP007sWT and mutants (SP007s*carA*$^-$, SP007s*carB*$^-$, SP007scar*AB*$^-$, SP007s*phl*$^-$&*plt*$^-$, SP007s*rpoS*$^-$) and purified CPSase on Xag growth (A), growth of SP007sWT and its mutants sprayed on plants 6 h before Xag 12−2 (B), and competitive colonization on leaves of biocontrol agents (7-SP007s strains) and causal pathogen (Xag 12−2). Different letters indicate significant differences as determine by DMRT ($P \leq 0.05$). (C)

## CONCLUSION

The CPSase activity in *P. fluorescens* SP007s was harbored with two genes, *carA* and *carB* corresponding for small CPSase subunit A and large subunit B synthesis respectively. The complete DNA sequences of these two genes exhibited extensive similarity to the corresponding genes of other *Pseudomonas* species. The transcription of *carA* or *carB* in SP007s had ability to synthesize CPSase independently by itself in its homologous background but their messenger RNA correlated closely with one another that they were found to be contiguous synthesis for the entire concentration of CPSase subunit A or subunit B. The *carAB* function in SP007s demonstrated a new mechanism in regulating CPSase biosynthesis products that could improve the effectiveness of biocontrol activity by degrading a virulence factor DSF of *X. axonopodis* pv. *glycines*. A close correlation was observed between higher concentration of CPSase and reduction level of disease symptoms, that probably leads to utilize a purified CPSase as decrease drug resistance and increased plant health from bacterial infection.

The organization of the genes within the region of the SP007s chromosome cloned in *carAB* affected the activity connecting to broad regulatory functions of extracellular protein synthesis. They included reduction of its growth and motility, protease, extracellular polysaccharides, biofilm development, antibiotics 2,4-DAPG (*phl*), pyoluteorin (*plt*), phenazine (*phzE*), pyochelin siderophore (*pch*) and sigma factor S (*rpoS*). These corresponding genes seem to be regulated by *carAB* indicating that strain SP007s reduced symptoms of BP disease on soybean plant was not only dependent on CPSase mechanism but also by other biocontrol biosynthesis. Thus, *carAB* might link to be more complex and employ of any additional regulatory gene function. Further investigation to clarify this hypothesis will provide much-needed insight into biocontrol mechanism of *carAB* regulation that will serve as the possibility of increased-biocontrol effectiveness in diverse-environment conditions.

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