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3 Title: **PsyR, a transcriptional regulator in quorum sensing system, binds *lux* box-like**

4 **sequence in *psyI* promoter without AHL quorum sensing signal molecules and activates**

5 ***psyI* transcription with AHL in *Pseudomonas syringae* pv. *tabaci* 6605**

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19

Abstract (248 words)

Quorum sensing (QS) is a mechanism for bacterial cell–cell communication using QS signals. *N*-acyl-homoserine lactones (AHL), QS signals in *Pseudomonas syringae* pv. *tabaci* (*Pta*) 6605, are synthesized by an AHL synthase (PsyI) and recognized by its cognate transcription factor PsyR. To reveal the role of PsyR in virulence, we generated Δ*psyR* mutant and complemented strains in *Pta* 6605. Thus, we found that the Δ*psyR* mutant is remarkably reduced in AHL production and ability to cause disease and propagate in host tobacco leaves. The phenotypes of complemented strains were restored to that of the wild-type (WT). Because the Δ*psyR* mutant lost nearly all AHL production, we investigated the function of PsyR in the transcription of *psyI* and AHL production. Electrophoretic mobility shift assays suggested that the recombinant PsyR protein binds the promoter region of *psyI* but not *psyR* without AHL. The addition of AHL did not significantly affect this binding. The binding core sequence of this region was identified as a 20 bp *lux* box-like sequence. To reveal the function of PsyR and AHL on *psyI* transcription, we constructed a *psyI* promoter::*lacZYA* chimeric reporter gene, and transformed it into the WT and Δ*psyI* mutant of *Pta* 6605. The β-galactosidase activity was increased in a bacterial density-dependent manner in the WT and also in a Δ*psyI* mutant by the addition of exogenous application of AHL. These results indicate that the solo PsyR binds the *lux* box in the *psyI* promoter and activates transcription in the concomitant presence of AHL.

Key words: AHL; PsyI; PsyR; quorum sensing

Introduction

Quorum sensing (QS) is a system for bacterial cell–cell communication using small diffusible signal molecules, which are also called QS signals or autoinducers. Many Gram-negative bacteria such as members of the genera *Erwinia*, *Vibrio*, *Pantoea*, and *Pseudomonas* use *N*-acyl-homoserine lactones (AHL) as QS molecules (Ham 2013; Tsai and Winans 2010; von Bodman et al. 2003). AHLs are synthesized by the coupling of homoserine lactone rings from S-adenosylmethionine and acyl chains from the acyl-acyl carrier protein (acyl-ACP) pool in cells by the AHL synthase enzymes, such as LuxI in *Vibrio fischeri* (Whitehead et al. 2001), EsaI in *Pantoea stewartii* (Beck von Bodman and Farrand 1995), and PsyI (or also called AhlI or PsmI) in *Pseudomonas syringae* (Elasri et al. 2001; Taguchi et al. 2006; Quiñones et al. 2004). *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL) is one of the most commonly reported molecules of quorum sensing signals. The concentration of OHHL increases as bacterial density increases. Thus, in *V. fisheri,* the molecular complex of transcription factor LuxR with OHHL binds to a promoter of the *luciferase* operon, *luxI*, which encodes AHL synthase and bioluminescence-related proteins, and activates its transcription when the concentration of OHHL reaches a threshold (Urbanowski et al. 2004). QS controls a wide variety of bacterial behaviors such as bioluminescence, swarming motility, biofilm formation, multidrug resistance, production of extracellular enzymes, antibiotics, epiphytic fitness, and altogether affects bacterial virulence (Fuqua et al. 2001; von Bodman et al. 2003).

In the best known QS system in *V. fischeri*, LuxR-type transcription factors dimerize and with AHL the complex binds to target promoters to activate transcription. However, it is also reported that another LuxR-type transcription factor, EsaR of *Pantoea stewartii*, a causative agent of Stewart's wilt of sweet corn and leaf blight of maize, negatively controls its own transcription, but its cognate AHL synthase gene *esaI* is not regulated by EsaR (von Bodman et al. 2003). EsaR proteins dimerize, bind the promoter region of *esaR* itself and the genes involved in exopolysaccharide (EPS) production in the absence of AHL to inhibit

1 transcription as a repressor (Beck von Bodman and Farrand 1995; Carlier et al. 2009;
2 Minogue et al. 2002). However, it was reported that EsaR in the absence of AHL also
3 functions as a transcriptional activator for small regulatory RNA, *esaS* (Schu et al. 2009). The
4 EsaR controls divergently transcribed genes *esaR* and *esaS*. The EsaR binding site is located
5 at a position centered around -10 of *esaR* and a position centered around -60 of *esaS.* EsaR
6 binding in the absence of AHL repressed transcription of *esaR*, but activated that of *esaS*.
7 Therefore, EsaR is an activator when binding site located at a position around -60, whereas
8 EsaR is a repressor when binding site located at a position around -10. In the presence of
9 AHL EsaR was released from the DNA, and the transcription of *esaR* was derepressed and
10 that of *esaS* was silenced (Schu et al. 2009; Shong et al. 2013; von Bodman et al. 2003). Thus,
11 the function of EsaR is changed by the binding site in the promoter of target genes and the
12 existence of AHL as a transcriptional activator and repressor.
13 It is also known that YenR, quorum sensing transcription factor in *Yersinia*
14 *enterocolitica*, activates transcription of small noncoding RNA gene, *yenS*, and the *yenS*
15 RNA involves the regulation of AHL production (Tsai and Winans 2011). In both *P. stewartii*
16 and *Y. enterocolitica* a set of genes, *esaI* and *esaR*, and *yenI* and *yenR*, are divergently
17 transcribed. Furthermore, *esaS* in *P. stewartii* and *yenS* in *Y. enterocolitica* lie adjacent to and
18 divergent from each *esaR* and *yenR*, respectively.
19 *P. syringae* is a Gram-negative bacterial pathogen that causes leaf spot, stem canker on
20 a wide range of plant species. Species of *P. syringae* is subdivided into about 50 pathogen
21 varieties (pathovars) based on their host plant species and type of disease symptoms.
22 Multilocus sequence analysis revealed that *P. syringae* species were divided into five clades
23 (Studholme 2011). Role of QS and regulation of AHL biosynthesis in *P. syringae* were
24 mainly investigated in *P. syringae* pv. *syringae* (*Pss*) B728a, a causal agent of brown spot on
25 bean which belongs to clade 2b (Quiñones et al. 2004) and *P. syringae* pv. *tabaci* (*Pta*) 6605,
26 a causal agent of tobacco wild fire disease which belongs to clade 3 (Kawakita et al. 2012;

1 Marutani et al. 2008; Taguchi et al. 2006). *Pss* B728a produces mainly OHHL as QS
2 molecule by AHL synthases AhlI, the product of *ahlI*, whereas *Pta* 6605 produces OHHL and
3 *N*-hexanoyl-L-homoserine lactone (HHL) as major QS molecules by AHL synthase PsyI, the
4 product of *psyI*. It was thought that each LuxR type transcription factor AhlR in *Pss* B728a
5 and PsyR in *Pta* 6605 together with AHL regulates each transcription of *ahlI* and *psyI*,
6 respectively. A *psyI* and *ahlI* genes, and *psyR* and *ahlR* genes are orthologues each other, and
7 amino acid identities of PsyR and AhlR, and PsyI and AhlI are high at 93% and 84%, and
8 their similarities are 100% and 98%, respectively. Both set of genes, *psyI* and *psyR* or *ahlI*
9 and *ahlR,* are transcribed convergently and responsible for AHL production as well as *esaI*
10 and *esaR* in *P. stewartii* and *yenI* and *yenR* in *Y. enterocolitica.* However, there is a neither
11 similar sequence to *esaS* of *P. stewartii* nor *yenS* of *Y. enterocolitica*. Thus, transcriptional
12 regulation of *psyI* by PsyR or *ahlI* by AhlR remains unclear.

13 In *Pss* B728a, expression of *ahlI* increased in a cell-density-dependent manner, and the
14 expression of *ahlI* was remarkably reduced in both *gacA* and *aefR* mutants (Quiñones et al.
15 2004). The GacS/GacA two-component system is one of perception system for environmental
16 signals in Gram-negative bacteria (Newton and Fray, 2004). The GacS is a sensor kinase
17 which autophosphorylate upon signal perception, and activates its partner, response regulator
18 GacA. Whereas, TetR family transcription factor, AefR functions as AHL production and
19 epiphytic fitness regulator (Quiñones et al. 2004). We previously investigated AHL
20 production-deficient mutant strains in *Pta* 6605 such as a *psyI*-defective mutant (Taguchi et al.
21 2006), Gac two-component-system defective mutants (Marutani et al. 2008), and a Δ*aefR*
22 mutant (Kawakita et al. 2012). Furthermore, we found that flagellar motility-defective mutant
23 strains such as Δ*fliC* (Taguchi et al. 2010) and Δ*motABCD* (Kanda et al. 2011) also impaired
24 AHL production. Recently, we found that the MexEF-OprN multidrug efflux pump
25 transporter negatively controls AHL accumulation in *Pta* 6605 (Sawada et al. 2018).
26 However, it is not known how *psyI* transcription is regulated in *P. syringae* species. As a first

1 step to revealing the mechanism of AHL production and the QS system in *P. syringae*, we
2 generated a Δ*psyR* mutant in *Pta* 6605 and examined its virulence. We further produced a
3 recombinant PsyR protein, and the specific binding of PsyR to target DNA was investigated.
4 Finally, we generated a system for monitoring *psyI* transcription using a *lacZYA* reporter gene,
5 and measured cell density-dependent and AHL-regulated *psyI* transcription. Based on these
6 analyses we discuss the role of PsyR in the regulation of gene expression and bacterial
7 virulence.
8
9 **Materials and methods**
10 **Bacterial strains and growth conditions**
11 All bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas*
12 *syringae* pv. *tabaci* 6605 strains were maintained in King's B (KB) medium at 27°C, and
13 *Escherichia coli* strains were grown at 37°C in Luria–Bertani (LB) medium.
14 *Chromobacterium violaceum* CV026 was grown at 30°C in LB medium with kanamycin at a
15 final concentration of 50 μg/ml (McClean *et al*. 1997).
16
17 **Plant material, inoculation procedure and bacterial growth**
18 Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi NC) were grown at 25°C with a 12 h
19 photoperiod. For dip-inoculation, bacterial strains were suspended in 10 mM $MgSO_4$ and
20 0.02% (v/v) Silwet L77 (OSI Specialties, Danbury, CT) at an OD_{600} of 0.1, and detached
21 tobacco leaves (three leaves for each strain) were soaked in that solution for 20 min. The
22 leaves were incubated under conditions of 85% humidity in a growth cabinet for 11 days at
23 23°C with an 18 h photoperiod. To maintain the turgidity of the detached leaves, water was
24 supplied to the cut petiole. To examine the bacterial growth in inoculated tobacco leaves, five
25 leaf disks (8 mm diameter) were punched from tobacco leaves and soaked in a bacterial
26 suspension in 10 mM $MgSO_4$ and 0.02% (v/v) Silwet at an OD_{600} of 0.1 for 20 min. The disks

1 were soaked in 15% (v/v) H_2O_2 for 1 min to sterilize the leaf surface 2 and 6 days after
2 inoculation, then were washed with sterile distilled water and ground in a mortar with a pestle.
3 Serially diluted samples (10 μl) in 10 mM $MgSO_4$ were spotted on KB plates, and the
4 numbers of colonies that appeared after 48 h incubation at 27°C were counted, and the
5 bacterial populations were calculated.
6
7 **Generation of *psyI* and *psyR* mutant strains and *psyR*-complemented strain**
8 To generate Δ*psyR* mutant of *Pta* 6605, each upstream and downstream region of *psyR* was
9 amplified with sets of primers, psyR-5F and psyR-5R for the upstream region and psyR-3F
10 and psyR-3R for the downstream region, by PCR using genomic DNA as a template (Fig. 1a,
11 Table 2). Each PCR-amplified DNA fragment was digested at the artificial *Bam*HI site
12 designed at the 5' end of psyR-5R and psyR-3F. After ligation of the appropriate upstream
13 and downstream fragments, PCR was carried out with psyR-5F and psyR-3R primers to
14 amplify ca. 1.8 kb DNA fragment, then inserted into a pCR Blunt II TOPO vector (Thermo
15 Fisher Scientific, Waltham, MA, USA) to obtain pCR Blunt-Δ*psyR*. The DNA fragment
16 possessing both upstream and downstream regions of *psyR* was excised by *Eco*RI digestion
17 and inserted into a mobilizable cloning vector pK18*mobSacB* (Schäfer et al. 1994) to obtain
18 pK18-Δ*psyR*. On the other hand, to generate the Δ*psyI* mutant, *psyI* and its surrounding
19 region was amplified by PCR using a set of PCR primers, psyI-1 and psyI-2 (Table 2); dAMP
20 (deoxyadenosine monophosphate) was added to the 3' end of the PCR product with 10 ×
21 A-attachment mix (Toyobo, Osaka, Japan), and then inserted into a pGEM-T Easy vector
22 (Promega, Madison, WI, USA). Using a recombinant plasmid DNA, pGEM-*psyI*, as a
23 template, inverse PCR was carried out using a set of PCR primers, psyI-3 and psyI-4, to
24 delete the *psyI* open reading frame. Then the PCR product and template DNA were digested
25 with *Xba*I and *Dpn*I. The resultant DNA was self-ligated with a Ligation-convenience kit
26 (Nippon Gene, Tokyo, Japan). The Δ*psyI* DNA construct was introduced into the *Eco*RI site

1 of pK18*mobsacB* to obtain pK18-Δ*psyI*. The pK18-Δ*psyR* and pK18-Δ*psyI* were introduced
2 into *E*. *coli* S17-1, and the Δ*psyR* and Δ*psyI* deletion mutants of *Pta* 6605 were generated by
3 conjugation and homologous recombination according to the method described previously
4 (Taguchi et al. 2006).
5 For complementation of the Δ*psyR* mutant, primers psyR-1 and psyR-2 (Fig. 1a, Table
6 2) were used to amplify the predicted promoter region and entire *psyR* ORF, and cloned in
7 pGEM-T Easy vector to obtain pGEM-*psyR*. The cloned DNA fragment was excised by
8 *Eco*RI digestion, and inserted into the *Eco*RI site of pBSL118, a transposon vector (Alexeyev
9 et al. 1995), to generate pBSL-*psyR*, and introduced into the Δ*psyR* mutant by conjugation
10 using the *E*. *coli* S17-1 λpir strain to generate *psyR*-C. All sequences of amplified DNA
11 fragments were determined by an ABI PRISM 3100 using a BIG Dye terminator cycle
12 sequencing kit (Thermo Fisher Scientific).
13
14 **Detection of *N*-acyl homoserine lactones**
15 Bacterial strains were grown in LB medium with 10 mM $MgCl_2$ or KB medium for 24 h at
16 27°C. AHLs extracted with an equal volume of ethyl acetate were detected using C_{18}
17 reversed-phase thin layer chromatography (TLC Silica gel 60, Merck, Darmstadt, Germany)
18 and the biosensor *C. violaceum* CV026 (Taguchi et al. 2006). As the standard molecules,
19 chemically synthesized *N*-hexanoyl-L-homoserine lactone (HHL, Sigma-Aldrich, St. Louis,
20 MS, USA) and *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL, Sigma-Aldrich) were used.
21
22 **Production of recombinant PsyR protein**
23 To produce recombinant PsyR protein (rPsyR), the *psyR* coding region was amplified with a
24 set of PCR primers (psyR-F and psyR-R, Table 2), and inserted into pMALc5X-His (New
25 England Biolabs, Ipswich, MA, USA) at *Nde*I and *Eco*RI sites to generate pMAL-psyR. The
26 recombinant plasmid pMAL-psyR was introduced into *E. coli* BL21, and rPsyR was

1 produced according to the manufacturer's instructions as fusion protein with maltose binding
2 protein (MBP). The rPsyR was further purified using a column filled with amylose resin.
3 Purified rPsyR was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
4
5 **Electrophoretic mobility shift assay of PsyR**
6 The interaction of rPsyR with the upstream region of *psyI* or *psyR* was assessed by an
7 electrophoretic mobility shift assay (EMSA). DNA fragments were prepared by PCR with
8 sets of primers (psyI-PF and psyI-PR for *psyI* and psyR-PF and psyR-PR for *psyR*) or
9 annealing of sets of oligonucleotides (Table 2), and DIG-labeled according to the DIG Gel
10 Shift Kit 2nd Generation (Sigma-Aldrich). An EMSA reaction mixture (20 μl) was prepared
11 according to the manufacturer's instructions. After electrophoresis, protein-DNA complexes
12 were transferred onto a Hybond-N+ membrane (GE Healthcare Biosciences, Piscataway, NJ,
13 USA), and detected with an anti-DIG antibody conjugated with alkaline phosphatase (Roche,
14 Basel, Switzerland) and CSPD as a chemiluminescence substrate (Thermo Fisher Scientific).
15 The chemiluminescence was detected with ChemiDoc Touch (Bio-Rad Laboratories,
16 Hercules, CA, USA).
17
18 **Construction of reporter plasmid**
19 A transposon vector pBSL118 digested at *Eco*RV and a general-purpose plasmid vector
20 pHSG396 (Takara Bio, Kusatsu, Japan) digested at *Hin*cII were ligated to produce pBSLC2,
21 then the *lacZYA* fragment was excised from pARO-*lacZYA* (Tamura et al. 2005) by *Bam*HI
22 and *Eco*RI digestion and inserted into pBSLC2 to create pBSLC2-*lacZYA* (Fig. 1b). The *psyI*
23 promoter region obtained by PCR with a set of primers psyI-F and psyI-R (Table 2) was
24 digested with *Bam*HI and *Spe*I, and inserted into the same restriction sites of pBSLC2-*lacZYA*
25 to generate pBSLC2-*psyI*-*lacZYA*.
26

1 **β-galactosidase activity to monitor *psyI* expression**

2 The β-galactosidase activity was measured as described by Miller (1992) with some
3 modification. The *psyI*::*lacZYA* chimeric reporter gene was introduced into a WT strain to
4 investigate cell density-dependent AHL production and Δ*psyI* strain to investigate the effect
5 of exogenous application of AHL; then β-galactosidase was measured. Promoter-less *lacZYA*
6 was introduced into WT as a negative control. Values are expressed as the means of at least
7 three independent experiments.

8

9 **Results and Discussion**

10 **Phenotype of Δ*psyR* mutant**

11 The AHL production in the WT, Δ*psyR* mutant, and its complemented strain was examined
12 using *C. violaceum* CV026, the aforementioned AHL biosensor strain (Taguchi et al. 2006).
13 The Δ*psyR* mutant strain greatly reduced the level of AHL production, but the complemented
14 strain restored it to the WT level (Fig. 2).

15 To investigate the virulence of the Δ*psyR* mutant strain, we carried out dip-inoculation
16 using tobacco leaves with WT, the Δ*psyR* mutant, and its complemented strain *psyR*-C of *Pta*
17 6605. As shown in Fig. 3a, the disease symptoms developed in the inoculation with WT and
18 *psyR*-C strains, whereas severe symptoms did not appear after inoculation with the Δ*psyR*
19 mutant strain. The bacterial growth in tobacco leaf disks was consistent with the development
20 of disease symptoms, and the population of the Δ*psyR* mutant was lower than that of other
21 strains at 2 and 6 days after inoculation (Fig. 3b), although there is no significant difference
22 in their in vitro growth (Fig. S1). These results clearly suggested that the virulence of the
23 Δ*psyR* mutant was remarkably reduced, but that of the *psyR*-C strain was recovered. Thus,
24 AHL-mediated quorum sensing system is important for virulence in *Pta* 6605. This result is
25 consistent with the previous result that the Δ*psyI* mutant had remarkably reduced virulence
26 (Taguchi et al. 2006).

1

2 **Specific binding of recombinant PsyR to a promoter of *psyI***

3 The rPsyR protein was produced using *E. coli*, and specific binding to the promoter of
4 potential target genes, *psyI* and *psyR*, was investigated by electrophoretic mobility shift assay
5 (EMSA, Fig. 4a). Purified rPsyR (280 nM or 480 nM) was incapable of shifting the promoter
6 of *psyR*; however, shifted bands (C1 and C2) appeared in the promoter of *psyI*. Although, the
7 identity of the two shifted bands is not clear, we expect that C1 seems to be composed of one
8 rPsyR and DNA, and C2 seems to be composed of two rPsyR and DNA. Because most
9 members of LuxR protein family function as dimer (Stevens et al. 2011). The dissociation
10 constant (*Kd*) of the PsyR for *psyI* promoter was roughly estimated as 70 nM (Fig. S2). To
11 investigate the effect of AHL on binding, different concentrations and combinations of
12 OHHL and HHL were mixed in a binding mixture of rPsyR and *psyI* promoter (Fig. 4b).
13 However, addition of AHL did not abolish the binding and had no apparent effect. Because
14 the rPsyR protein contains MBP at N-terminus, it is possible that MBP interfered the access
15 of AHL to the original N-terminal domain of PsyR which has AHL binding properties (Fuqua
16 et al., 2001). Although our attempt to purify intact rPsyR protein by removing MBP domain
17 was failed, we could generate the Δ*psyR* strains which possess fusion gene for MBP and
18 PsyR. The complemented strains by fusion gene for MBP/psyR evidently restored AHL
19 production (Fig. S3), indicating that the fusion protein MBP/PsyR is functional in nature.
20 Therefore, it is evident that PsyR without AHL bound to the promoter of *psyI*. AHL might
21 make binding stronger or change the conformation of PsyR to transcriptionally active form.
22 In the promoter region of the genes that are regulated by quorum sensing, *lux* box-like
23 sequences are conserved as binding sites for transcriptional regulators such as LuxR. The *lux*
24 box was first identified in the promoter of *luxI* in *V. fischeri* ATCC7744 (Devine et al., 1988).
25 The *lux* box-like sequences consist of 20 bp (core in Fig. 5) and are imperfect inverted
26 repeats. The *lux* box-like sequences also found in the *esaR* promoter of *Pantoea stewartii*

1 (Minogue *et al*., 2002), *psmI* promoter of *P*. *syringae* pv. *maculicola* strain CFBP 10912–9
2 (Elasri et al., 2001), and *psyI* promoter of *Pta* 6605 (Sawada et al. 2018, Fig. 5b). To confirm
3 the specific binding of PsyR to the *lux* box-like sequence, an EMSA competition assay was
4 carried out. A 153 bp fragment of the *psyI* promoter was DIG-labeled, and the *lux* box-like
5 sequence-containing fragments and related DNA fragments were used as competitors (Fig.
6 5a). As shown in Fig. 5c, addition of the *lux* box-like sequence containing DNA fragments
7 (60 and core) to an EMSA reaction mixture competed completely, and shifted bands
8 disappeared. However, this competition was not found by the addition of the DNA fragments
9 that did not contain *lux* box-like sequence (5'-20 and 3'-20, Fig. 5c). Although a slight
10 competition was observed by the addition of 60m, it might be due to the mutated sequence of
11 60m (5 repeats of ACGT that is possible to make inverted repeat). These results clearly
12 suggest that the *lux* box-like sequence plays a central role in this binding.
13
14 **Transcriptional regulation of *psyI* promoter by PsyR and AHL**
15 Incubation of *Pta* 6605 WT possessing a chimeric reporter gene *psyI*::*lacZYA* was started at a
16 variety of cell densities (OD_{600} = 0.01, 0.1, 0.5, and 1.0), and the β-galactosidase activities
17 were measured after 4 h. As shown in Fig. 6a, the β-galactosidase activity was low when
18 bacteria started incubation at 0.01 or 0.1 cell densities, whereas it became higher at 0.5 or 1.0
19 cell densities. Although high cell density activated β-galactosidase activity, this effect was
20 nearly saturated at 0.5 cell density. The *psyI*::*lacZYA* was also introduced into Δ*psyI* mutant.
21 The β-galactosidase activity of the Δ*psyI* mutant was low without application of AHL, but it
22 increased with the application of AHL in a dose-dependent manner (Fig. 6b). These results
23 indicate that *psyI* transcribed at a constitutively low level without AHL, but it will be
24 activated with AHL.
25 We observed that the *psyR* gene expression in the Δ*psyI* mutant was reduced to 1/3
26 level in wild type strain (Taguchi et al. 2015). The *psyR* gene expression decreased as well as

1 *psyI* in other AHL production-defective mutants such as Δ*fliC*, Δ*fliD*, Δ*motABCD*, Δ*fgt1* and
2 Δ*aefR* (Taguchi et al. 2015). Although it is reported that transcription of *psyI* and *psyR* are
3 independently regulated in *Pss* B728a (Quiñones et al. 2004), expression of *psyR* is high
4 when *psyI* is actively transcribed and low when not (Taguchi et al. 2015). Further, exogenous
5 application of AHL did not induce *psyI* expression in Δ*psyR* mutant (Fig. S4). These results
6 indicate that transcription of *psyI* requires PsyR and AHL, and transcription of *psyI* and *psyR*
7 cooperatively activates at high density-cells.

8 The LuxR protein family was subdivided into five different classes based on
9 interactions of the LuxR homologues with AHL, DNA-binding activity, and their multimeric
10 properties (Stevens et al. 2011). Among them, classes 1–3, including LuxR type transcription
11 factors, are transcriptional activators; for example, LuxR was reported to bind the *luxI*
12 promoter with AHL (Urbanowski et al. 2004), class 4, such as EsaR type transcriptional
13 regulators, bind target DNA without AHL as repressors, and class 5 regulators do not
14 dimerize in response to AHL, and the AHL they recognize is produced from neighboring
15 cells; there is no partner LuxI homologue. PsyR of *Pta* 6605 is similar to classes 1–3 as a
16 transcriptional activator. However, unlike classes 1–3, PsyR binds its target DNA without
17 AHL, and activates transcription with AHL. Therefore, PsyR belongs to a novel class of
18 transcriptional regulator. Recently, we reported that MexEF-OprN, a multidrug efflux pump
19 transporter, negatively regulates AHL accumulation (Sawada et al. 2018). In this research
20 process, we also clarified that a *lux* box-like sequence is essential for the activation of *psyI*
21 transcription, because the mutant of *Pta* 6605 with *lux* box-deleted *psyI* promoter is impaired
22 in AHL production. This result also supports the hypothesis that PsyR is a transcriptional
23 activator and the *lux* box is a positive element for *psyI* transcription.

24

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5
6 **Compliance with ethical standards**
7 **Conflicts of interest**
8 The authors declare that they have no conflict of interest.
9 **Ethical approval**
10 This article does not contain any studies with human participants or animals performed by
11 any of the authors.
12
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1 **Figure legends**

2 **Fig. 1** Generation of mutant, complemented strains, and reporter plasmid. (a) Schematic
3 organization of *psyI*, *psyR*, and the surrounding region in *Pseudomonas syringae* pv. *tabaci*
4 6605 and construction of Δ*psyR* and its complemented strain and Δ*psyI* mutant. PCR was
5 carried out to isolate each DNA fragment to generate mutant or complemented strains using
6 respective primers indicated by arrows. (b) Structure of *psyI* reporter plasmid,
7 pBSLC2-*psyI-lacZYA*. Plasmid is not drawn to scale. The region between "I" and "O" is
8 randomly inserted into the bacterial genome. The *oriV* and ColE1 *ori* are replication origins
9 of R6K and colicin E1 plasmids, respectively, and *oriT* is an RP4 plasmid-derived
10 conjugative transfer origin. The *tnp* encodes transposase. Amp, ampicillin; Cm,
11 chloramphenicol; Km, kanamycin; E/H, *Eco*RV/*Hin*cII.

12 **Fig. 2** AHL production. *N*-hexanoyl-L-homoserine lactone (HHL) and
13 *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) are visualized by violacein production
14 using *C. violaceum* CV026.

15 **Fig. 3** Inoculation of host tobacco leaves by *Pta* 6605 WT and Δ*psyR* mutant and its
16 complemented strains by a dip method. (a) Tobacco leaves were dip-inoculated with each
17 bacterial suspension (OD_{600} = 0.1 in 10 mM $MgSO_4$) for 20 min at 23°C. Photographs taken
18 11 d after inoculation show representative results obtained from three independent
19 experiments. (b) Bacterial population was calculated at 0, 2, and 6 d post inoculation. The
20 bars represent standard deviations for three independent experiments. Asterisks indicate a
21 significant difference from the WT (*$P<0.01$).

22 **Fig. 4** Electrophoretic mobility shift assays (EMSA) of the rPsyR to the upstream
23 promoter-containing regions. (a) EMSA of rPsyR to the *psyI* and *psyR* promoters. The *psyI*
24 promoter (8.8 fmol) and the *psyR* promoter (4.6 fmol) were DIG-labeled, then incubated with
25 0, 280, and 480 nM of rPsyR. (b) Effect of AHL on the binding of rPsyR to the labeled *psyI*

1 promoter (8.8 fmol). Various concentrations of HHL and/or OHHL were added to the binding
2 mixture. F denotes a free DNA probe, C1 and C2 denote DNA-rPsyR complexes.
3 **Fig. 5** Identification of binding site of rPsyR on *psyI* promoter. (a) DIG-labeled DNA probe
4 and series of DNA competitors. Sizes and location of DNA fragments are illustrated.
5 Numbers of the DNA fragments refer to the position of the nucleotides relative to the "A" of
6 translation start codon ATG. The dotted line indicates a mutated sequence. (b) Conserved *lux*
7 box-like sequences in the *psyI* promoter in *Pta* 6605 and *Psm*, *luxI* promoter in *V. fischeri*,
8 and *esaR* promoter in *P. stewartii*. The nucleotides identical to the *lux* like-box in *Pta* 6605
9 are indicated in black. The imperfect inverted repeat is indicated with arrows. (c)
10 Competition assay of binding between 480 nM of rPsyR and labeled *psyI* promoter (8.8 fmol).
11 Each non-labeled DNA (40 pmol) was added into the binding reaction mixture as competitors.
12 The reaction mixtures were separated on 10% (w/v) polyacrylamide gel.
13 **Fig. 6** Promoter activity of *psyI* in *Pta* 6605. The *psyI* promoter was fused to the *lacZYA* gene,
14 and β-galactosidase activities are indicated as Miller units. (a) WT *Pta* 6605 possessing the
15 *psyI* promoter with a *lacZYA* reporter gene was incubated for 4 h to avoid the effect of
16 preculture conditions with a different cell inoculum (OD_{600} = 0.01, 0.1, 0.5, and 1.0). The
17 effect of bacterial cell density on β-galactosidase activity was examined. Each symbol
18 indicates the result of an independent experiment. (b) Effect of exogenous application of
19 AHL on *psyI* expression in the Δ*psyI* mutant. Bacterial incubation was started at OD_{600} =
20 0.015 with or without different concentrations of OHHL and HHL. The β-galactosidase
21 activity was measured after 24 h incubation. Values are expressed as the means of at least
22 three independent experiments.

Table 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Reference or source
E. coli strain		
DH5α	*F*$^-$, *λ*$^-$, *ø80dLacZΔM15*, Δ*(lacZYA-argF)*U169, *recA1*, *endA1*, *hsdR17*($r_K^- m_K^+$), *supE44*, *thi-1*, *gyrA*, *relA1*	Takara Bio
S17-1	*Thi*, *pro*, *hsdR*$^-$, *hsdM*$^+$, *recA* [chr::RP4-2-Tc::Mu-Km::Tn7]	Schäfer *et al.* (1994)
S17-1 λpir	λ *pir* lysogen of S17-1	Simon *et al*. (1983)
BL21	*F*$^-$, *ompT*, *hsdS*$_B$($r_B^- m_B^-$), *gal*, *dcm*	New England Biolabs
Chromobacterium violaceum CV026	Double mini-Tn*5* mutant from *C. violaceum* ATCC 31532; AHL biosensor	McClean *et al*. (1997)
P. syringae pv. *tabaci*		
Isolate 6605	Wild-type, Nal^r	Taguchi et al. (2006)
Δ*psyR*	Isolate 6605 Δ*psyR*, Nal^r	This study
psyR-C	Isolate 6605 Δ*psyR* (*psyR*), Nal^r, Km^r	This study
Δ*psyI*	Isolate 6605 Δ*psyI*, Nal^r	This study
Promoter less	promoter less::*lacZYA-introduced 6605*, Nal^r, Km^r	This study
WT *psyI* reporter	Wild-type 6605 possessing *psyI*::*lacZYA*, Nal^r, Km^r	This study
Δ*psyI psyI* reporter	Δ*psyI* possessing *psyI*::*lacZYA*,, Nal^r, Km^r	This study
Plasmid		
pCR Blunt II TOPO	pCR Blunt II TOPO cloning vector, Km^r	Thermo Fisher Scientific
pCR Blunt-Δ*psyR*	pCR Blunt II TOPO with 858-bp upstream and 956-bp downstream region of *psyR*, Km^r	This study
pGEM-T Easy	Cloning vector, Amp^r	Promega
pGEM-*psyI*	pGEM-T Easy possessing 2603 bp including *psyI*, Amp^r	This study
pGEM-Δ*psyI*	pGEM-T Easy possessing upstream and downstream region of *psyI*, Amp^r	This study
pGEM-*psyR*	pGEM possessing 965-bp *psyR* with promoter region, Amp^r	This study
pK18*mobsacB*	Small mobilizable vector, Km^r, sucrose-sensitive (*sacB*)	Schäfer *et al.* (1994)
pK18-Δ*psyR*	pK18mobsacB with 858-bp upstream and 956-bp downstream region of *psyR*, Km^r	This study
pK18-Δ*psyI*	pK18mobsacB with *psyI*-deleted DNA fragment, Km^r	This study
pBSL118	Mini-Tn5 derived plasmid vector for insertion mutagenesis, Amp^r, Km^r	Alexeyev *et al.* (1995)
pBSL-*psyR*	pBSL118 possessing 965-bp *psyR* with promoter region, Amp^r, Km^r	This study
pMAL-c5X-His	Protein expression vector with maltose-binding protein, Amp^r	New England Biolabs
pMAL-psyR	pMAL-c5X-His with 741 bp of *psyR* open reading frame	This study
pHSG396	General purpose plasmid vector, Cm^r	Takara Bio
pBSLC2	Transposon vector constructed by ligation of *Eco*RV-digested pBSL118 and *Hin*cII-digested pHSG396, Amp^r, Km^r, Cm^r	This study

pARO-*lacZYA*	pARO191 possessing *lacZYA*	Tamura et al. 2005
pBSLC2-*lacZYA*	pBSLC2 possessing *lacZYA*	This study
pBSLC2-*psyI-lacZYA*	pBSLC2-*lacZYA* possessing *psyI* promoter	This study

Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance, Km^r, kanamycin resistance, Nal^r, nalidixic acid resistance

Table 2 Oligonucleotide sequences used in this study.

Oligonucleotide name	Sequence (5'-3')	Purpose
psyR-5F	AGCCTGCGTCTCGCTTAA	PCR amplification of upstream region of *psyR* for cloning
psyR-5R	gggatccTGGCTGTACTCATTATCCGC	
psyR-3F	cgggatccTGACTCAGGCAGCGCTGTG	PCR amplification of downstream region of *psyR* for cloning
psyR-3R	CAGGAACCGCGTTCAAGGTG	
psyI-1	TGGTGTCTTGTAGCGGCCAG	PCR amplification of *psyI* and surrounding region for cloning
psyI-2	AATCCCATCCGGTGTGCGTG	
psyI-3	gctctagaGAAACTCAAACCCGCTCGACAT	Inverse PCR to delete *psyI* coding region to generate Δ*psyI*
psyI-4	gctctagaCTGAGTCAGACCATGCCCAT	
psyR-1	ACAAAATCCCATCCGGTGTG	PCR amplification of *psyR* with promoter region for complementation
psyR-2	TCAGACCATGCCCATGTTGAT	
psyR-F	ccatatgGAGGTTCGTACCGTGAAAGC	PCR amplification to introduce *psyR* into pMALc5X-His
psyR-R	ggaattcGACCATGCCCATGTTGATGGC	
psyI-PF	AGAACGCCGAAGCAGATTTC	PCR to prepare *psyI* DNA probe (153 bp) for EMSA
psyI-PR	CCTTGCACAACCAGTGTGAA	
psyR-PF	CACTACTCCTTGCTGTCGGA	PCR to prepare *psyR* DNA probe (290 bp) for EMSA
psyR-PR	GTAATACCTTGTTTATTGTTTGTTCTCGG	
60-S	TGTTATATTTCAAGGTGTTGACCTGTTCTTAGGTACAGTAGATTTACCGTTTGAAATACG	DNA probe for EMSA
60-AS	CGTATTTCAAACGGTAAATCTACTGTACCTAAGAACAGGTCAACACCTTGAAATATAACA	
60m-S	TGTTATATTTCAAGGTGTTGacgtacgtacgtacgtacgtGATTTACCGTTTGAAATACG	DNA probe for EMSA
60m-AS	CGTATTTCAAACGGTAAATCacgtacgtacgtacgtacgtCAACACCTTGAAATATAACA	
5'-20-S	TGTTATATTTCAAGGTGTTG	DNA probe for EMSA
5'-20-AS	CAACACCTTGAAATATAACA	
Core-S	ACCTGTTCTTAGGTACAGTA	DNA probe for EMSA
Core-AS	TACTGTACCTAAGAACAGGT	
3'-20-S	GATTTACCGTTTGAAATACG	DNA probe for EMSA
3'-20-AS	CGTATTTCAAACGGTAAATC	
psyI-F	ggactagtACCATTATCCCTATGGAGTCAT	PCR to prepare *psyI* promoter for construction of reporter plasmid
psyI-R	cgggatccTACGTAACGGGCATCGTCGTG	

Underlined letters indicate the translation start codon in psyR-F and the antisense for translation stop codon of *psyR* in psyR-2. Small letters indicate "additive nucleotides" containing artificial *Bam*HI sites in psyR-5R, psyR-3F and psyI-R, *Nde*I site in psyR-F, *Eco*RI site in psyR-R, *Xba*I sites in psyI-3 and psyI-4 and *Spe*I site in psyI-F, respectively. Small letters in 60m-S and 60m-AS indicate mutated sequence.

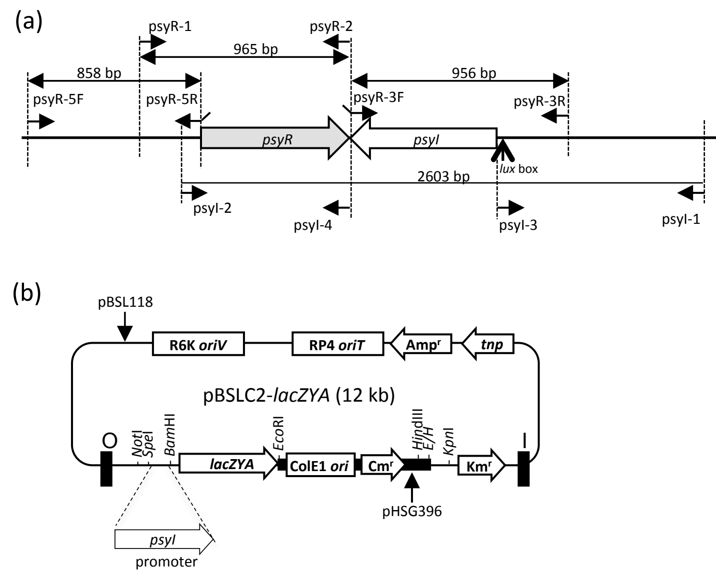


Fig. 1. Generation of mutant, complemented strains, and reporter plasmid. (a) Schematic organization of *psyI*, *psyR*, and the surrounding region in *Pseudomonas syringae* pv. *tabaci* 6605 and construction of Δ*psyR* and its complemented strain and Δ*psyI* mutant. PCR was carried out to isolate each DNA fragment to generate mutant or complemented strains using respective primers indicated by arrows. (b) Structure of *psyI* reporter plasmid (pBSLC2-*psyI*-*lacZYA*). Plasmid is not drawn to scale. The region between "I" and "O" is randomly inserted into the bacterial genome. The *oriV* and ColE1 *ori* are replication origins of R6K and colicin E1 plasmids, respectively, and *oriT* is an RP4 plasmid-derived conjugative transfer origin. The *tnp* encodes transposase. Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; E/H, *Eco*RI/*Hin*cII.

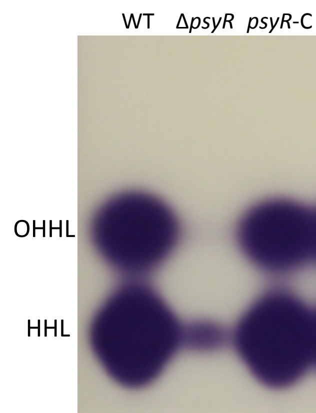
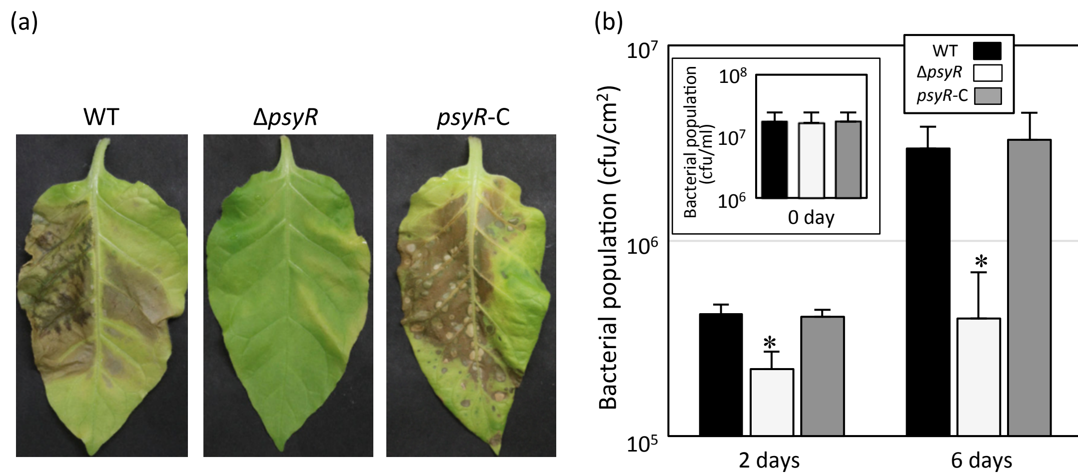


Fig. 2. AHL production. *N*-hexanoyl-L-homoserine lactone (HHL) and *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) are visualized by violacein production using *C. violaceum* CV026.



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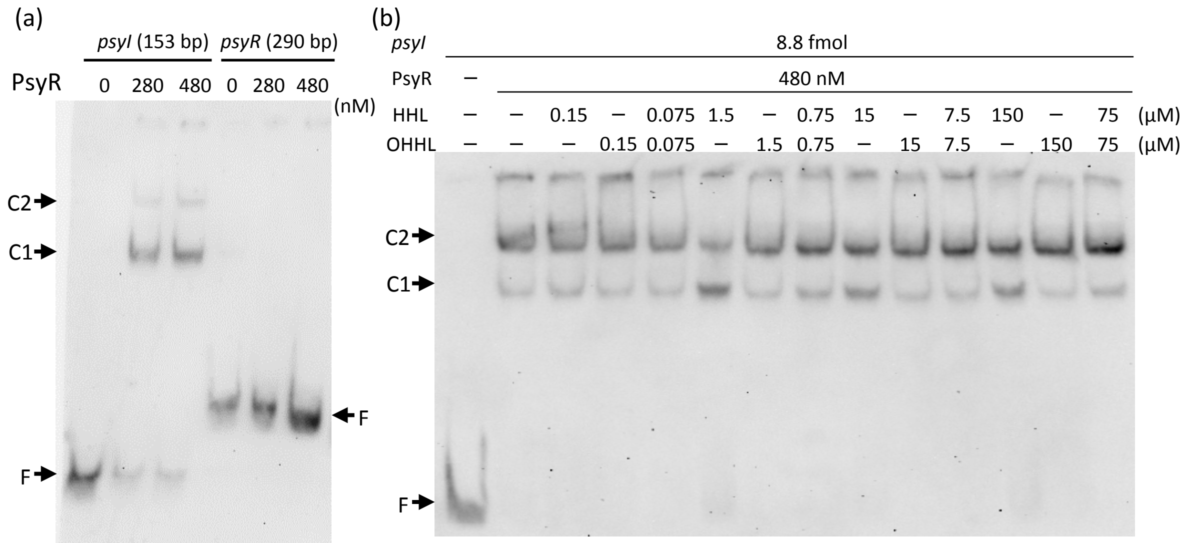

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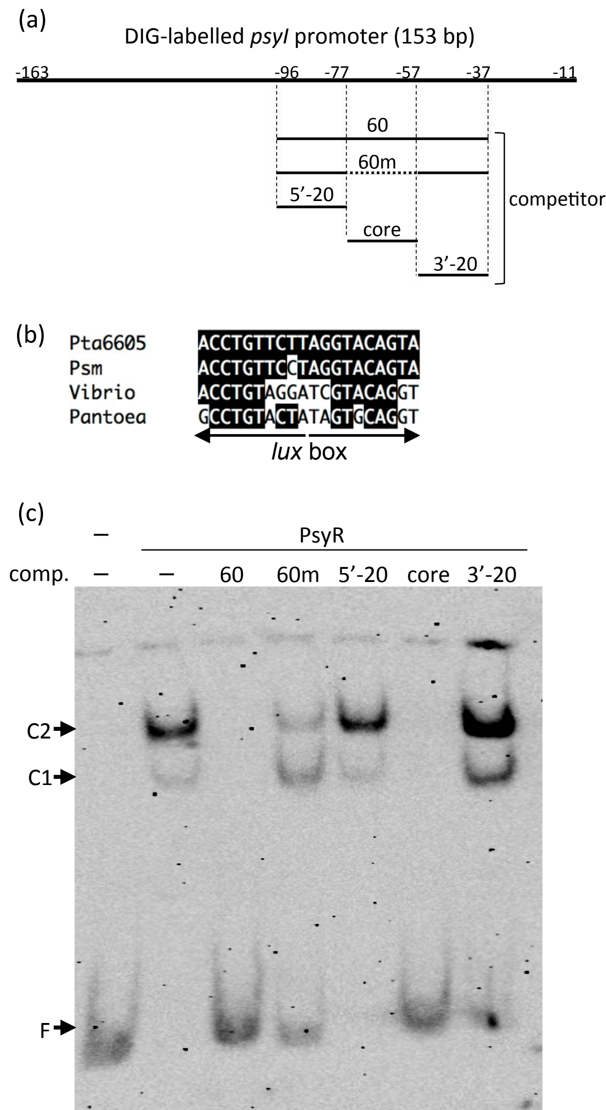

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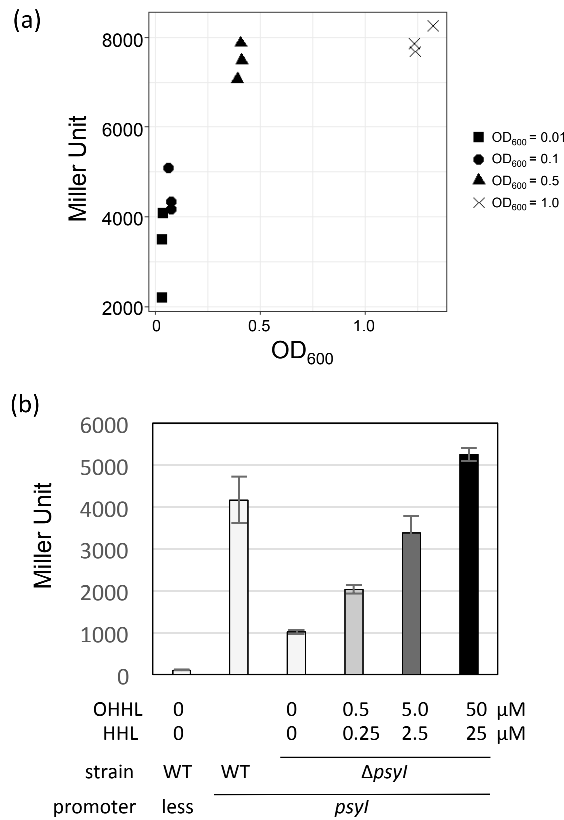

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