Available online at www.sciencedirect.com
SCIENCE DIRECT
Research in Microbiology 157 (2006) 169–175
Research in Microbiology
www.elsevier.com/locate/resmic

Identification of an HptB-mediated multi-step phosphorelay in *Pseudomonas aeruginosa* PAO1

Ching-Ting Lin [1], Ying-Jung Huang [1], Pei-Hsuan Chu, Jye-Lin Hsu, Chih-Hsiang Huang, Hwei-Ling Peng *

Department of Biological Science and Technology, National Chiao Tung University, 75 Po Ai Street, Hsin Chu 30050, Taiwan, Republic of China

Received 23 March 2005; accepted 23 June 2005

Available online 7 September 2005

Abstract

We herein demonstrate that the hybrid sensor PA1611 carries out specific signal transduction, through HptB (PA3345), to the response regulator PA3346 in *Pseudomonas aeruginosa* PAO1. As assessed by phenotypic changes in the *hptB* deletion mutant, the pathway is likely to be involved in the regulation of flagellar activity, the chemotaxis response, twitching motility, and biofilm formation in the bacteria.

Keywords: HptB; Hybrid sensor PA1611; *Pseudomonas aeruginosa* PAO1

1. Introduction

Two-component systems (2CS) are frequently involved in aiding the adaptation of bacteria to changing environmental conditions. In general, a bacterial 2CS is composed of a sensor kinase, to detect one or several environmental stimuli, and a response regulator, to be phosphorylated by the sensor kinase, thereby regulating the expression of necessary response phenotypes [9]. Based on organization of their functional domains, 2CS can be classified into three major types: the classical system, the unorthodox system, and the hybrid system [16]. The sensor of a typical hybrid system is an ITR-type histidine kinase carrying *i*nput-, *t*ransmitter- and *r*eceiver-domains. It is speculated that an ITR sensor normally requires a separate histidine-containing phosphotransfer (Hpt) molecule to relay the signaling phosphate from sensor to the corresponding response regulator [16].

Annotation of the *Pseudomonas aeruginosa* PAO1 genome (http://www.pseudomonas.com/) has revealed 123 2CS encoding genes including 12 ITR-type sensor kinase and 3 Hpt-encoding genes [2,16]. Unlike most bacterial 2CS gene pairs in which the sensor gene and the regulator gene are closely linked in the genome, the 3 Hpt encoding genes are located separately from any of the ITR sensor genes in the *P. aeruginosa* genome. Thus, it is not clear whether any of these Hpt molecules could serve as the target of the ITR sensors, nor which ITR sensor and Hpt could form cognate pairs. Based on genome annotation and BLASTX analysis, the 12 ITR-type sensor kinases can be classified into six functional groups: Na^+/proline symporters (PA1396, PA1976, PA1992 and PA3271), an ABC-type amino acid transport/signal transduction system (PA2583), MmoS homologs (PA4586 and PA2824), FixL homologs (PA1243 and PA2177), GacS homologs (PA1611 and PA3462), and a hypothetical protein (PA3974). PA1611 was chosen arbitrarily to investigate whether it really is capable of transferring the phosphate to an Hpt molecule. In this study, we demonstrate a specific phosphorelay from hybrid PA1611 through one of the three Hpt modules, HptB (PA3345) to the response regulator PA3346.

2. Materials and methods

2.1. Strains, plasmids, growth media, and culture condition

The bacterial strains and plasmids used in this study are listed in Table 1. The growth media used were LB (Luria broth)

* Corresponding author.
E-mail address: hlpeng@cc.nctu.edu.tw (H.-L. Peng).
[1] The authors contributed equally to this work.

0923-2508/$ – see front matter
doi:10.1016/j.resmic.2005.06.012

Table 1
Bacterial strains and plasmids used in this study

Strains or plasmids	Descriptions	Reference or source
Strains		
P. aeruginosa		
PAO1	Non-mucoid *P. aeruginosa* strain O1	Laboratory stock
MPA45	*P. aeruginosa* strain O1 Δ*hptB* Km^r	This study
OPA45	Strain MPA45 carrying pBM2	This study
MPA45C	Strain MPA45 carrying pMMB66	This study
Plasmids		
pGEM-T	Ap^r; PCR cloning vector	Promega
pET30a-b	His-tag protein expression vector, Km^r	Novagen
pSUP202	Tc^r; suicide vector	[19]
pMMB66	Ap^r; broad-host-range expression vector	[8]
pT16HD	Ap^r; 1.7-kb fragment of PA1611HD PCR amplified using primer pair, 1611-5 and 1611-3, cloned into pGEM-T	This study
pE1611HD	Km^r; 1.7-kb *Nco*I/*Sal*I digested fragment of pT16HD cloned into pET30a	This study
pTHptA	Ap^r; 0.5-kb fragment of *hptA* PCR amplified using primer pair, A5 and A3, cloned into pGEM-T	This study
pEHptA	Km^r; 0.6-kb *Sac*I/*Hin*dIII digested fragment of pTHptA cloned into pET30c	This study
pTHptB	Ap^r; 0.5-kb fragment of *hptB* PCR amplified using primer pair, 3345-5 and 3345-6, cloned into pGEM-T	This study
pEHptB	Km^r; 0.6-kb *Sac*I-digasted fragment of pTHptB cloned into pET30b	This study
pTHptC	Ap^r; 0.4-kb fragment of *hptC* PCR amplified using primer pair, C5 and C3, cloned into pGEM-T	This study
pEHptC	Km^r; 0.4-kb *Sac*I/*Not*I digested fragment of pTHptC cloned into pET30b	This study
pT46R	Ap^r; fragment of PA3346 receiver domain PCR amplified using primer pair, 3346-R1 and 3346-R2, cloned into pGEM-T	This study
pE46R	Km^r; *Bam*HI/*Hin*dIII digested fragment of pT46R cloned into pET30a	This study
pBM2	Ap^r; the *hptB* containing fragment from pEHptB subcloned into pMMB66	This study

or minimal medium plus appropriate antibiotics. Bacteria were grown at 37 °C unless otherwise indicated.

2.2. Expression vector construction

The coding regions of PA1611HD, *hptA*, *hptB*, *hptC*, and PA3346R were amplified from *P. aeruginosa* PAO1 chromosomal DNA using the primer pairs (Table 2) and the PCR products were cloned into the pGEM-T vector. Each of the coding regions contained in the resulting plasmids pT16HD, pTHpatA, pTHptB, pTHptC, and pT46R were then subcloned respectively using appropriate restriction enzymes into pET30 expression vectors for the production of PE1611HD, pEHptA, pEHptB, pEHptC, and PE46R (Table 1).

2.3. Expression and purification of the histidine-tagged proteins

The resulting plasmids were transformed respectively into *E. coli* NovaBlue (DE3). The bacteria containing each of the plasmids were cultured in LB to log phase, and induced by adding 0.5 mM or 1 mM IPTG, with incubation at 37 °C for 4 h. The IPTG-induced bacterial cells were harvested by centrifugation and then disrupted by sonication. The N-terminal His-tag facilitated purification of recombinant PA1611HD, HptA, HptB, HptC, and PA3346R by using a nickel charged resin (Novagen, Madison, WI). Finally, these proteins were concentrated with PEG 20 000 and protein concentrations were determined by the Bradford assay [1].

Table 2
Primers used in this study

Primers (protein)	Sequence (5′ → 3′)
1611-5 (1611HD)	5′-TTC ATC AAG CTT CGC GAC CG-3′
1611-3 (1611HD)	5′-TTG CAG ATC CAT GGC GGC-3′
A5 (HptA)	5′-CGC GCA GTG AGC TCC CTT TA-3′
A3 (HptA)	5′-GAC GCG AAG CTT ACG GGC TC-3′
3345-5 (HptB)	5′-CGC GGA GCT CCT ATT GGG TA-3′
3345-6 (HptB)	5′-CGC CAC CCT CGA GTA CCC G-3′
C5 (HptC)	5′-CCG CCA CGA GCT CAT ATC A-3′
C3 (HptC)	5′-TGG ACC TGT CGA CCC TGG A-3′
3346-R1 (PA3346)	5′-GCA GTT GTT CAG GAT CCG CT-3′
3346-R2 (PA3346)	5′-AAT GGG CAA GCT TGT CGA AC-3′

2.4. In vitro phosphorelay assay

The conditions for the phosphorylation assays were as described [15]. Briefly, 1 μg the purified recombinant protein was incubated with 2.5 μCi of [γ-P^{32}]ATP in phosphorylation buffer, and the mixture was incubated at 25 °C for 1 h for the autokinase activity. Aliquots of the labeled sensor protein were added to 1 μg of the purified HptA, HptB, and HptC, respectively, and the reaction mixtures were incubated further for 20 min at 37 °C. In order to show that HptB as a mediator is able to accelerate the transfer of the phosphoric group to the likely cognate regulator PA3346R, 1 μg of PA3346R was added to the mixture containing phosphorylated PA1611HD and HptB, and the incubation continued at 37 °C for 20 min. All the reaction mixtures were stopped by adding an equal volume of SDS–PAGE loading buffer (50 mM Tris–Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), separated by 17.5% SDS–PAGE and detected by InstantImager™ (Packard Instrument Company).

2.5. Construction of the hptB mutant

To generate an *hptB* deletion in *P. aeruginosa*, the fragments of approximately 1 kb in length flanking both sides of *hptB* were PCR-amplified by appropriate primer pairs and cloned into the suicide vector pSUP202 [19]. The DNA fragment containing a kanamycin-resistant gene PCR amplified from pET30a was then inserted into the pSUP202-derived plasmid. The resulting plasmid was transformed subsequently into *E. coli* S17-1/*-pir* by electroporation. The transformants in mid-log phase were then mixed with *P. aeruginosa* PAO1 at a ratio of 1:5 and the mixture spotted on a sterile nitrocellulose membrane attached to the LB agar plate. After overnight incubation at 37 °C, the cells were washed off the membrane with 4 ml of LB. The transconjugants were selected by plating with a 10^4-fold dilution of the culture on LB agar plates containing 100 μg/ml tetracycline for integration of the plasmid. The tetracycline resistant conjugants were then cultured in LB to late logarithmic phase followed by selection on either LB–Tc plates (100 μg/ml tetracycline) or LB–Km plates (300 μg/ml kanamycin) for loss of the plasmid. The mutant MPA45 carrying the *hptB* deletion was confirmed further by PCR and Southern blot analysis. The *hptB* gene was also PCR-amplified and the DNA cloned into the broad host range plasmid pMMB66 [8], which resulted in plasmid pBM2. The *hptB* mutation was complemented by transferring pBM2 into strain MPA45 and the transformants were selected by carbenicillin 200 μg/ml.

2.6. Swimming assay

Tryptone swimming plates were composed of 0.3% bactoagar, 0.5% NaCl, and 1% tryptone. Bacteria were inoculated with a sterile toothpick and incubated for 48 h at 30 °C [5].

2.7. Chemotaxis response assay

The chemotaxis response was measured by the capillary assay. Essentially, a 1-ml tuberculin syringe with a disposable 25-gauge needle was filled with 100 μl of Bushnell–Hass (BH) mineral salts medium with or without 0.1% tryptone as a chemoattractant [5]. Bacteria cultured overnight were diluted ten-fold to an $OD_{600\ nm} = 0.2$ and the cells were replenished and grown in LB at 37 °C to logarithmic phase, pelleted and resuspended in BH. 100 μl of the bacterial suspension was drawn into a 200 μl pipette tip and the syringe was inserted into the pipette tip with the neck of the needle forming a tight fit with the base of the tip. The construct was then incubated at 37 °C for 45 min. The content of the syringe was then diluted in PBS and plated onto LB plates for cell enumeration.

2.8. Twitching motility assay

Bacteria were stab-inoculated with a toothpick through a thin LB agar layer (1% agar) to the bottom of the Petri dish. After incubation for 48 h at 30 °C, a hazy zone of growth at the interface between the agar and the polystyrene surface was observed [4]. The twitching capacity was examined by removing the agar, washing unattached cells with water, and staining the attached cells with 1% CV [5].

2.9. Quantitation of biofilm formation

Biofilm formation was assessed by the ability of the cells to adhere to the walls of 96-well microtiter dishes made of PVC (TPP Industries, France) with some modification of the reported protocol [7]. The indicator medium (100 μl/well) contained an aliquot of 1:50-diluted overnight bacteria culture, the concentration of which was adjusted approximately to $OD_{600\ nm} = 0.6$. The plates were incubated at 30 °C for 10 h for biofilm formation. 150 μl of a 1% solution of crystal violet (CV) was then added to each well, the plates were placed at room temperature for 15 min and rinsed thoroughly with water. The CV-stained biofilm was solubilized in 150 μl of 95% ethanol and the absorbance determined at 595 nm in a spectrophotometer.

3. Results and discussion

3.1. Identification of a multi-step phosphorelay pathway

PA1611 is annotated as being a homolog of GacS (LemA), the sensor of the Gac regulon which has been shown to be required for regulation of bacterial motility [11] and biofilm formation [13]. The protein tends to form an insoluble inclusion body on overexpression, presumably caused by the presence of the transmembrane domain. To eliminate the problem, the coding region containing only the cytoplasmic catalytic part of the protein including the histidine-containing transmitter (H) and aspartate-containing receiver (D) domains of PA1611 was cloned into expression vector pET30a to produce the recombinant protein designated as PE1611HD (Table 1). As listed in Table 1, the coding regions of the three Hpt encoding genes were also cloned into the pET expression vector for production of HptA (PA0991), HptB (PA3345), and HptC (PA0033) proteins respectively. The His-tag allowed purification of recombinant PA1611HD, HptB and HptC by affinity chromatography to homogeneity as shown in Fig. 1A. Nevertheless, a protein of approximately 26 kDa (Fig. 1A) appeared to be constantly co-purified with the HptA protein using different purification procedures. The protein was hence isolated, subjected to trypsin digestion and MALDI/TOF analysis. The resulting peptide profile was then analyzed using the Mascot program of ExPASY (http://www.matrixscience.com/search_form_select.html) and a database search. Interestingly, the co-purified protein, which is rather resistant to treatment with SDS, appeared to be an HptA dimer.

To verify whether PA1611HD possessed autokinase activity, phosphorylation assays was performed. As shown in Fig. 1B, PA1611HD clearly displayed autokinase activity. No signal of phosphorylation could be detected on recombinant HptA, HptB, and HptC, as expected. By incubating phosphorylated PA1611HD with each of the purified HptA, HptB and HptC, it

was observed that only HptB was able to receive the phosphate group (Fig. 2). This suggests that the hybrid sensor PA1611 is able to relay the signaling phosphate specifically to HptB.

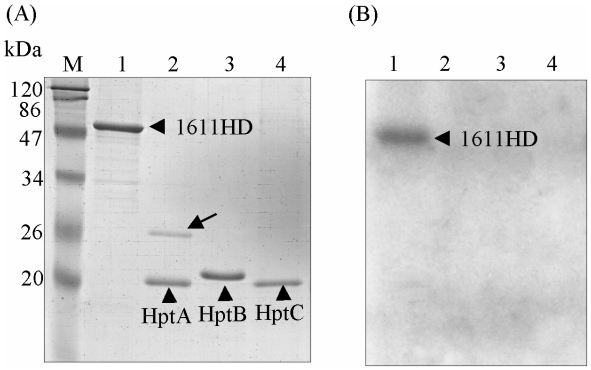

Fig. 1. Autokinase activity of PA1611HD. Approximately 1 μg of the tested recombinant proteins was individually incubated with [γ-^{32}P]-ATP (2.5 μCi) at 25 °C for 1 h and resolved on an SDS–polyacrylamide gel. Lane 1: PA1611HD; 2: HptA; 3: HptB; 4: HptC. The gel was stained with Coomassie blue (A) or subjected to autoradiography (B). M stands for the molecular weight marker. The star represents the dimer form of HptA.

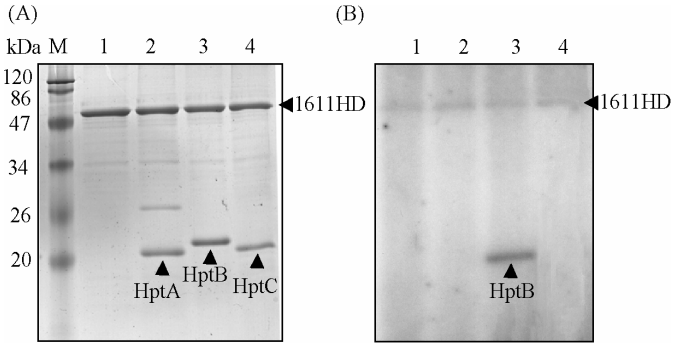

Fig. 2. In vitro phosphotransfer experiment on PA1611HD and the three Hpt molecules. PA1611HD was preincubated with [γ-^{32}P]-ATP at 25 °C for 1 h and then the phosphorelay was initiated by adding HptA (lane 1), HptB (lane 2), and HptC (lane 3), respectively. The reaction was continued for another 20 min at 37 °C, determined to be the most optimal temperature for the phosphorelaying reaction. The reaction mixtures were resolved by SDS–PAGE and the gel was stained with Coomassie blue (A) or subjected to autoradiography (B). The location of each recombinant protein is marked by an arrowhead. M stands for the molecular weight marker.

In order for the bacterium to respond to the signal appropriately, the phosphorylated HptB has to transfer the phosphate group to the corresponding response regulator and thus proceed with the signaling pathway. According to the Pseudomonas Genome Project (http://www.pseudomonas.com/), PA3346 encodes a protein with a typical receiver domain of a response regulator (from residues 14 to 127). Since functionally coupled genes are often clustered in bacterial genomes [14], we hypothesize that PA3346, located next to *hptB*, is likely to be the cognate response regulator for receiving the signal relay from HptB. To facilitate recombinant protein production in *E. coli*, we cloned the region comprising the receiver domain of PA3346 from residues 1–165 into the pET vector and the recombinant protein PA3346R which was fused with His-tag at the N-terminus was purified from the *E. coli* transformant. As shown in Fig. 3, PA3346R did not display any autokinase activity. Nor did incubation of PA3346R with PA1611HD display any signal of phosphorylation. However, when both HptB and phosphorylated PA1611HD were present, PA3346R was found to be able to receive the phosphate group. The results suggest that phosphorylated PA1611HD could not transfer the phosphate group directly to PA3346R, implying the requirement for an HptB mediator in the phosphorelay pathway.

3.2. *Construction and characterization of the* hptB *mutants*

Using rpsblast analysis, in addition to the receptor domain, PA3346 also contains a PP2C domain (from residues 185–567; Fig. 4A) of *Bacillus subtilis* RsbU. RsbU, a serine phosphatase, has been shown to regulate the expression of sigma B positively through dephosphorylation of the anti-sigma B antagonist RsbV [22]. Upstream from *hptB* and PA3346, PA3347 is a homolog of anti-sigma factor antagonist RsbV and SpoIIAA, which could be dephosphorylated by SpoIIE serine phosphatase [18]. As an RsbU homolog, PA3346 likely performs a similar regulatory role by dephosphorylating the anti-sigma factor antagonist PA3347. It has been reported that the flagellar biosynthesis genes and chemotaxis genes of *P. aeruginosa* PAO1 are clustered into three regions on the chromosome [3]. As shown in Fig. 4B, the region III for flagella biogenesis [3] was found upstream of PA3347 which contains the genes en-

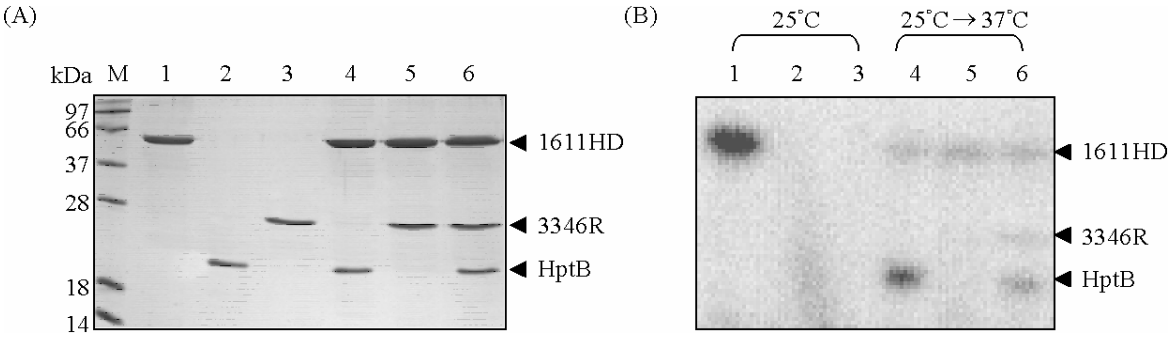

Fig. 3. In vitro phosphotransfer experiment of PA1611HD, HptB and PA3346R. Approximately 1 μg each of PA1611HD (lane 1), HptB (lane 2), PA3346R (lane 3) was incubated with [γ-^{32}P]-ATP (2.5 μCi) individually for 1 h at 25 °C. In addition, the phospho-PA1611HD was incubated in the presence of HptB (lane 4), PA3346R (lane 5) or both (lane 6) for 20 min at 37 °C. These reaction mixtures were separated by SDS–PAGE in duplicate and one of the gels was stained with Coomassie blue (A) while the other was detected by an InstantImager™ (Packard Instrument Company) (B). The locations of the recombinant proteins and phosphorelated proteins are indicated by arrowheads. M stands for the molecular weight marker.

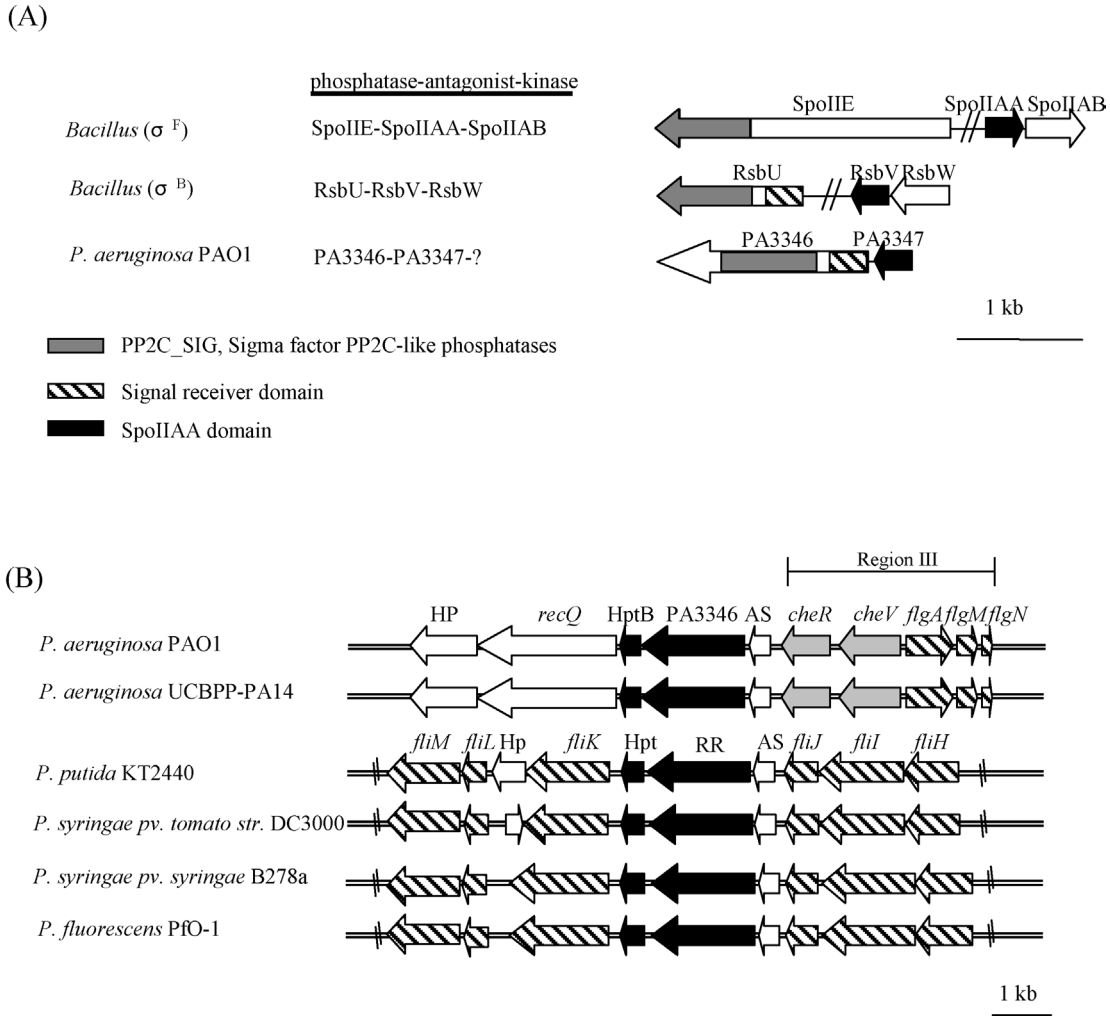

Fig. 4. The gene organization of the *hptB*-containing gene cluster. (A) The conserved domains, analyzed by rpsblast, of the phosphatase-antagonist-kinase cascade in *Bacillus* and *P. aeruginosa* PAO1 were compared. (B) The flanking genes of HptB were annotated according to either the released genome of *P. aeruginosa* PAO1 (http://www.pseudomonas.com/) and NCBI or by BLASTX analysis. The proximal genes with known or predicated function involved in the same system are indicated with the same arrow bars. The arrow represents the direction of transcription of the genes. The proteins to which each gene was assigned are shown above the arrows. HP: hypothetical protein; AS: anti-anti-sigma factor; RR: response regulator. Region III consists of genes coding for parts of the flagellar export apparatus, antisigma factor FlgM and additional chemotaxis regulatory proteins [3].

coding regulatory proteins CheV (PA3348) and CheR (PA3349) for bacterial chemotaxis, *flgA* (PA3350) and *flgN* (PA3352) encoding flagellar assembly proteins, and *flgM* (PA3351) encoding anti-sigma F factor. Most interestingly, we have found that the gene cluster *hptB*-3346-3347 is also contained in other *Pseudomonas* species including *P. aeruginosa* UCBPP-PA14, *P. putida* KT2440, *P. syringae* pv. *tomato* str. DC3000, *P. fluorescens* PfO-1 and *P. syringae* pv. *syringae* B278a (Fig. 4B). The conserved gene organization after speciation suggests a preserved functional pathway.

In order to investigate whether the multi-step signal transduction pathway played a role in flagellar motility and the chemotaxis response, an *htpB* deletion mutant was constructed using allelic exchange strategy. The deletion of the resultant mutant MPA45 was confirmed by Southern blot analysis using *hptB* as a probe. In addition, the plasmid pBM2 was transferred into *P. aeruginosa* MPA45, resulting in the complementation strain OPA45. While comparing the growth in LB medium of either MPA45 or OPA45 with that of wild-type PAO1, an identical growth curve was observed (data not shown). The biological properties including swimming activity, chemotaxis response and biofilm formation of the parental strain PAO1, *hptB* mutant MPA45 and the complementation strain OPA45 were also assessed comparably.

The polar flagellum of *P. aeruginosa* is responsible for swimming motility in an aqueous environment [5]. As shown in Fig. 5A, the *hptB* mutant MPA45 revealed reduced motility on the swimming plate. The reduced motility could be restored only in strain OPA45 which carries an *hptB*-expressing plasmid but not in MPA45C that harbors pMMB66 (Fig. 5A). This indicates that the disruption of the *hptB*-mediated signal transduction pathway affects bacterial swimming motility. It is well known that expression of flagellar motility involves the expression of more than 40 different genes which include the genes encoding regulatory proteins, structural components of flagellar secretion and assembly apparatus, and also proteins involved in

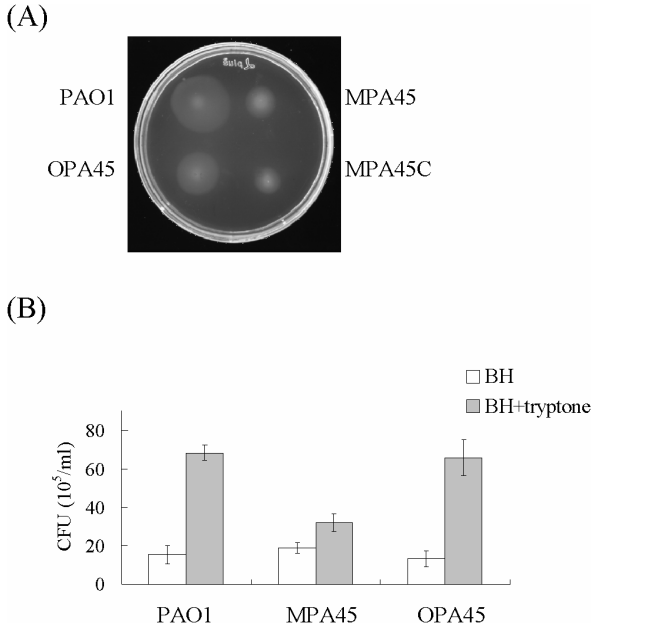

Fig. 5. Swimming activity assay and chemotaxis responses of PAO1, MPA45, OPA45, and MPA45C. (A) Swimming activity assay was performed as described [5]. The photograph was taken after 48 h of incubation of the bacteria on the swimming plate at 30 °C. MPA45C which is the bacteria MPA45 carrying pMMB66, was used as the control strain. (B) Capillary apparatus with or without 0.1% tryptone as a chemoattractant in Bushnell–Hass (BH) mineral salts medium inoculated with each of the bacteria was incubated at 37 °C for 45 min. The bacteria contained in the syringe were then plated onto LB plates for cell enumeration. Error bars represent the standard deviation of triplicates.

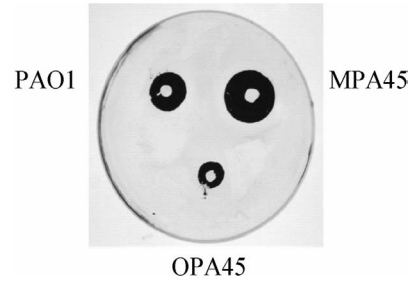

Fig. 6. Twitching motility of PAO1, MPA45, and OPA45. The twitching motility was assayed by inoculating the bacteria with a toothpick through LB agar (1% agar) to the bottom of the petri dish. After incubation of the bacteria at 30 °C for 48 h, the agar was then scraped off and stained with 1% crystal violet.

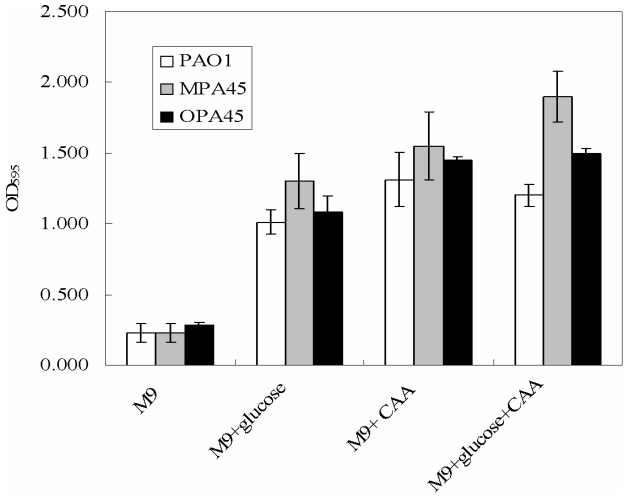

Fig. 7. Comparison of the biofilm formation of PAO1, MPA45, and OPA45. The cells were grown at 30 °C in minimal medium with each of the supplements including (1) 0.2% glucose; (2) 0.5% casamino acid (CAA); and (3) 0.2% glucose + 0.5% CAA. The extent of biofilm formation after 10 h of growth was stained by 1% CV (crystal violet) and expressed as the absorbance at 595 nm.

generating rotational motor force and chemosensory machinery [3]. To ascertain whether the change in bacterial motility is due to alteration of the flagellar structure, TEM (transmission electronic microscopy) analysis was then performed. However, the micrograph revealed no apparent difference in length and number of flagella between the mutant OPA45 and the parental bacteria (data not shown).

Over recent years, some chemosensory systems have been described as controlling bacterial motility [6]. The bacterial chemosensory system is composed of methyl-accepting chemotaxis proteins (MCPs) which are coupled via the adaptor protein CheW to the histidine protein kinase CheA. CheA transfers the phosphoryl group to the response regulator CheY, which then interacts directly with the motor to control the direction of flagellar rotation. The methylation status of the MCPs is adjusted via methyltransferase CheR and methyl esterase CheB [20]. CheV appears to be a composite protein [10] which possesses an N-terminal CheW sequence and a carboxyl-CheY domain. As shown in Fig. 5B, MPA45 displayed a reduced chemotaxis response. In contrast, *P. aeruginosa* OPA45 displayed a similar chemotactic activity as in the parental strain, indicating that the defect could be restored by complementing with a functional *hptB* gene. This result suggests that HptB-mediated multi-step phosphorelay has a role in regulating the chemosensory system in PAO1.

At the top of flagellar hierarchical regulation, alternative sigma factor FliA regulates at least 11 operons in *P. aeruginosa* [3]. It has been reported that expression of chemosensory genes (*cheAB*, *cheW*, *cheVR*) and flagella-associated genes (*motAB*, *fliCfleL*, and *flgMN*) was significantly reduced in the *fliA* deletion mutant [3]. Therefore, it is likely that decreased motility, as well as the decrease in the chemotaxis response in the *hptB* mutant, could be attributed to a less free form of sigma factor FliA. It was suggested previously that *fliA* transcription is initiated by an as yet uncharacterized sigma factor [3]. We herein hypothesize that, through control of the uncharacterized sigma factor, which is mediated by HptB (PA3345)-RsbU (PA3346)-anti-sigma factor antagonist (PA3347), functional FliA is able to regulate bacterial flagellar activity and the chemotaxis response. The hypothesis, however, remains to be demonstrated.

In addition to swimming, *P. aeruginosa* could move on a solid surface by twitching motility via the extension and retraction of type IV pili [12]. Interestingly, the *hptB* deletion mutant MPA45 exhibited a higher level of twitching motility in comparison with that of PAO1 (Fig. 6). This indicated that HptB also plays a role in controlling bacterial twitching motility.

Both expression of flagella and type IV pili appeared to be important for biofilm formation in *P. aeruginosa* [17]. Nutritional factors including casamino acids and glucose have been

demonstrated to be able to regulate biofilm development in the bacteria [21]. We were also interested in determining whether disruption of *hptB* would affect biofilm-forming activity. In this study, *P. aeruginosa* strains PAO1, MPA45 and OPA45 were inoculated into a 96-well tissue culture plate containing different nutritional factors in M9 medium, and biofilm formation was examined. As shown in Fig. 7, biofilm formation activity was found to increase in the *hptB* mutant only when bacteria were grown in M9 medium containing casamino acids and glucose, suggesting that HptB negatively regulates biofilm formation. It has been reported that the increase in availability of the carbon substrate may lead to a reduction in the surface-associated biofilm mass [7]. It is hence concluded that the HptB-mediated phosphorelay is likely to be involved in controlling biofilm formation upon changing the nutrient.

Taken together, these results indicate the existence of a specific phosphotransfer pathway from the sensor kinase PA1611 to HptB and then to PA3346. As assessed by phenotypic changes in the *hptB* deletion mutant, the pathway is likely involved in regulation of flagellar activity, the chemotaxis response, twitching motility and biofilm formation in bacteria.

Acknowledgements

We are thankful for the support of the National Science Council of the Republic of China (NSC92-2311-B009-001) to H.L.P. and are also grateful to Dr. Nandini for her critical reading of the manuscript.

References

[1] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[2] Y.T. Chen, H.Y. Chang, C.L. Lu, H.L. Peng, Evolutionary analysis of the two-component systems in *Pseudomonas aeruginosa* PAO1, J. Mol. Evol. 59 (2004) 725–737.

[3] N. Dasgupta, M.C. Wolfgang, A.L. Goodman, S.K. Arora, J. Jyot, S. Lory, R. Ramphal, A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*, Mol. Microbiol. 50 (2003) 809–824.

[4] A. Dazins, The *pilG* gene product, required for *Pseudomonas aeruginosa* pilus production and twitching motility, is homologous to the enteric, single-domain response regulator CheY, J. Bacteriol. 175 (1993) 5934–5944.

[5] E. Deziel, Y. Comeau, R. Villemur, Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities, J. Bacteriol. 184 (2001) 1195–1204.

[6] A. Ferrandez, A.C. Hawkins, D.T. Summerfield, C.S. Harwood, Cluster II *che* genes from *Pseudomonas aeruginosa* are required for an optimal chemotactic response, J. Bacteriol. 184 (2002) 4374–4383.

[7] M. Fletcher, The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene, Can. J. Microbiol. 23 (1977) 1–6.

[8] J.P. Furste, W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, E. Lanka, Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector, Gene 48 (1986) 119–131.

[9] J.A. Hoch, Two-component and phosphorelay signal transduction, Curr. Opin. Microbiol. 3 (2000) 165–170.

[10] E. Karatan, M.M. Saulmon, M.W. Bunn, G.W. Ordal, Phosphorylation of the response regulator CheV is required for adaptation to attractants during *Bacillus subtilis* chemotaxis, J. Biol. Chem. 276 (2001) 43618–43626.

[11] T.G. Kinscherf, D.K. Willis, Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl-homoserine lactone biosynthetic gene *ahlI*, J. Bacteriol. 181 (1999) 4133–4136.

[12] J.S. Mattick, Type IV pili and twitching motility, Annu. Rev. Microbiol. 56 (2002) 289–314.

[13] N. Ohta, A. Newton, The core dimerization domains of histidine kinase contain recognition specificity for the cognate response regulator, J. Bacteriol. 185 (2003) 4424–4431.

[14] M.D. Parkins, H. Ceri, D.G. Storey, *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation, Mol. Microbiol. 40 (2001) 1215–1226.

[15] V. Rangaswamy, C.L. Bender, Phosphorylation of CorS and CorR, regulatory proteins that modulate production of phytotoxin coronatine in *Pseudomonas syringae*, FEMS Microbiol. Lett. 193 (2000) 13–18.

[16] A. Rodrigue, Y. Quentin, A. Lazdunski, V. Mejean, M. Foglino, Two-component systems in *Pseudomonas aeruginosa*: Why so many?, Trends Microbiol. 8 (2000) 498–504.

[17] K. Sauer, M.C. Cullen, A.H. Rickard, L.A. Zeef, D.G. Davies, P. Gilbert, Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm, J. Bacteriol. 186 (2004) 7312–7326.

[18] T. Searls, X. Chen, S. Allen, M.D. Yudkin, Evaluation of the kinetic properties of the sporulation protein SpoIIE of *Bacillus subtilis* by inclusion in a model membrane, J. Bacteriol. 186 (2004) 3195–3201.

[19] R. Simon, J. Quandt, W. Klipp, New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria, Gene 80 (1989) 161–169.

[20] C.B. Whitchurch, A.J. Leech, M.D. Young, D. Kennedy, J.L. Sargent, J.J. Bertrand, A.B. Semmler, A.S. Mellick, P.R. Martin, R.A. Alm, M. Hobbs, S.A. Beatson, B. Huang, L. Nguyen, J.C. Commolli, J.N. Engel, A. Darzins, J.S. Mattick, Characterization of a complex chemosensory signal transduction system which controls twitching motility in *Pseudomonas aeruginosa*, Mol. Microbiol. 52 (2004) 873–893.

[21] J.W.T. Wimpenny, R. Colasanti, A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models, FEMS Microbiol. Ecol. 22 (1997) 1–16.

[22] A.A. Wise, C.W. Prise, Four additional genes in the *sigB* operon *of Bacillus subtilis* that control activity of the general stress factor σ^B in response to environmental signals, J. Bacteriol. 177 (1995) 123–133.