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FEMS Microbiology Letters, Vol. 231, 247-252 (2004)

Chemotaxis proteins and transducers for aerotaxis in *Pseudomonas aeruginosa*

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Abstract

It was previously shown that the chemotaxis gene cluster 1 (*cheYZABW*) was required for chemotaxis. In this study, the involvement of the same cluster in aerotaxis is described and two transducer genes for aerotaxis are identified. Aerotaxis assays of a number of deletion-insertion mutants of *Pseudomonas aeruginosa* PAO1 revealed that the chemotaxis gene cluster 1 and *cheR* are required for aerotaxis. Mutant strains which contained deletions in the methyl-accepting chemotaxis protein-like genes *tlpC* and *tlpG*, respectively, showed decreased aerotaxis. A double mutant deficient in *tlpC* and *tlpG* was negative for aerotaxis. TlpC has 45% amino acid identities with the *Escherichia coli* aerotactic transducer Aer. The TlpG protein has a predicted C-terminal segment with 89% identity to the highly conserved domain of the *E. coli* serine chemoreceptor Tsr. A hydropathy plot of TlpG indicated that hydrophobic membrane-spanning regions are missing in TlpG. A PAS motif was found in the N-terminal domains of TlpC and TlpG. On this basis, the *tlpC* and *tlpG* genes were renamed *aer* and *aer-2*, respectively. No significant homology other than the PAS motif was detected in the

N-terminal domains between Aer and Aer-2.

Keywords: Aerotaxis; *Pseudomonas aeruginosa*; Chemotaxis; Behavioral response; Transducer

1. Introduction

Pseudomonas aeruginosa is an obligately aerobic bacterium and is capable of swimming by rotating a single polar flagellum. This organism inhabits a wide range of environments, from soil and water to the human host. It is an opportunistic pathogen that is among the most frequently isolated bacteria in nosocomial infections [1]. *P. aeruginosa*, like most other motile bacteria, has chemotactic responses to a wide range of chemical stimuli. *P. aeruginosa* is attracted to 20 commonly occurring L-amino acids [2,3], sugars [4], organic acids [5], and inorganic phosphate [6]. *P. aeruginosa* is repelled by thiocyanic and isothiocyanic esters [7] and volatile chlorinated aliphatic compounds such as trichloroethylene, tetrachloroethylene, trichloroethane, and chloroform [8]. Chemotaxis is a clear indicator of bacterial responses to changing environments. It can be viewed as an important prelude to metabolism, symbiosis, and other ecological interactions in microbial communities [9].

Aerotaxis is the movement of a cell towards or away from oxygen. Bacteria use aerotaxis to swim toward an optimal oxygen concentration for their metabolism. Aerotaxis has been extensively investigated in the facultative anaerobe *Escherichia coli*. In *E. coli*, an active respiratory chain and chemotaxis (Che) proteins such as CheA, CheY, and CheW are required and Aer and Tsr function as independent sensor/transducers for aerotaxis [10-12]. Aer is a methyl-accepting chemotaxis protein (MCP)-like transducer which contains a PAS (an acronym of the *Drosophila* period clock protein [**P**ER], vertebrate aryl hydrocarbon receptor nuclear translocator [**A**RNT], and *Drosophila* single-minded protein [**S**IM]) motif in the N-terminal domain [13]. The PAS motif comprises a binding pocket for a prosthetic group [14] and Bibikov et al. [12] showed that Aer contained high levels of noncovalently associated flavin adenine dinucleotide (FAD). It is postulated that Aer uses FAD to monitor altered redox conditions in the cytoplasm. We recently demonstrated that *P. aeruginosa* also shows aerotaxis by using the chemotaxis well chamber method [8]. However, the mechanisms of aerotaxis

in *P. aeruginosa* are still poorly understood.

Analysis of the complete genome sequence of *P. aeruginosa* PAO1 suggested that the *P. aeruginosa* chemosensory system is very complex, with more than 20 *che* genes situated in 5 distinct clusters and 26 *mcp*-like genes scattered throughout the genome [15-17]. We demonstrated that *cheY*, *cheZ*, *cheA* , *cheB* , and *cheW* in Che cluster 1 (Fig. 1) and *cheR* in Che cluster 2 are responsible for chemotactic responses to amino acids and phosphate [18,19]. The *pilGHIJ* genes in Che cluster 3 are involved in twitching motility [20,21]. Of 26 MCP-like genes, only 6 genes (*pctA* , *pctB* , *pctC* , *ctpH* , *ctpL* , and *pilJ*) have been characterized to date [2,3,16,21]. The remaining 20 MCP-like genes and Che clusters 4 and 5 are as yet uncharacterized. In this study, we genetically analyzed aerotaxis in *P. aeruginosa* and found that *P. aeruginosa* possesses two chemoreceptors for aerotaxis, designated Aer and Aer-2. We also showed that Che cluster 1 and the *cheR* gene were required for aerotaxis.

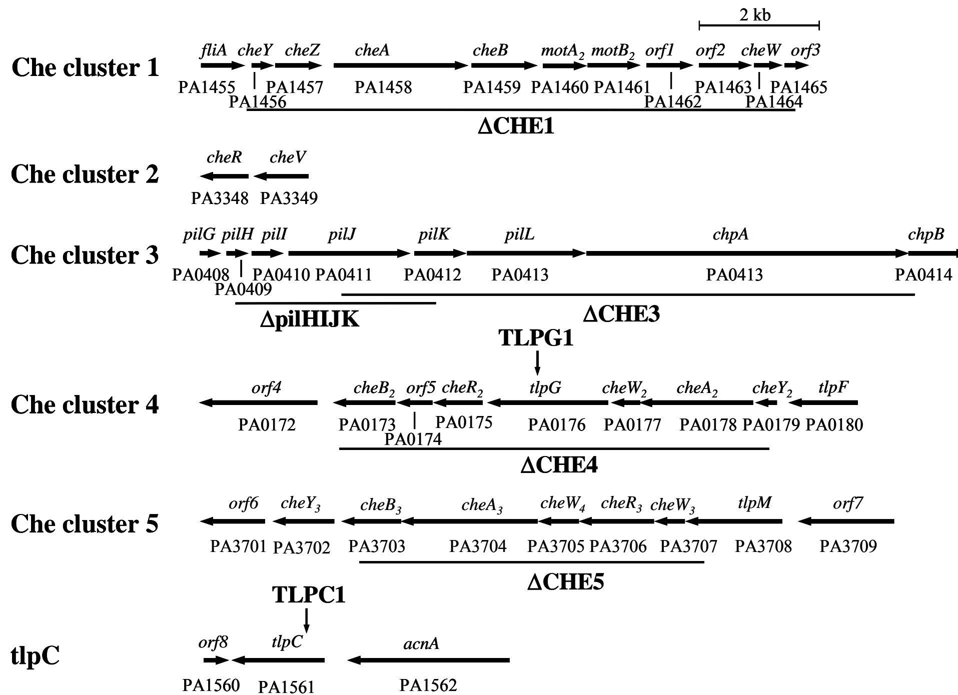

Fig. 1. Genetic organization of *che* clusters of *P. aeruginosa* PAO1. The locations and orientations of individual ORFs are shown by horizontal arrows. Gene ID numbers used in the *P. aeruginosa* genome sequencing project (http://www.pseudomonas.com/) are indicated below horizontal arrows. The locations of sequences deleted in deletion-insertion mutants (ΔCHE1, ΔpilHIJK, ΔCHE3, ΔCHE4, and ΔCHE5) are indicated by horizontal lines. Vertical arrows show the insertion sites of the *kan* and *tet* gene cassettes in TLPG1 and TLPC1, respectively.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 1. *E. coli* MV1184 and HB101, which were used for plasmid construction and DNA manipulation, were grown at 37˚C with shaking in 2 x YT medium [22] supplemented with appropriate antibiotics. This medium was also used for the preparation of *P. aeruginosa* cells for aerotaxis assays and electroporation.

Table 1
Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
MV1184	*ara* Δ(*lac*-*proAB*) *rpsL thi* (ϕ80 *lacZ*ΔM15) Δ(*srl*-*recA*)*306*::Tn*10*(Tc^r) F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	[31]
HB101	*hsdS20 recA13 proA2 leu6 thi-1 rpsL20 ara-14*	[32]
P. aeruginosa		
PAO1	Prototroph, FP^- (sex factor minus)	[33]
ΔCHE1	PAO1 derivative, ΔChe cluster 1 (*cheY cheZ cheA cheB* PA1460-1463 [b] *cheW*)	This study
ΔCHE3	PAO1 derivative, ΔChe cluster 3 (*pilJ pilK pilL chpA chpB*)	This study
ΔCHE4	PAO1 derivative, ΔChe cluster 4 ($cheY_2$ $cheA_2$ $cheW_2$ *tlpG* $cheR_2$ PA0174 [b] $cheB_2$)	This study
ΔCHE5	PAO1 derivative, ΔChe cluster 5 ($cheW_3$ $cheR_3$ $cheW_4$ $cheA_3$ $cheB_3$)	This study
ΔpilHIJK	PAO1 derivative, Δ*pilHIJK* cluster (*pilH pilI pilJ pilK*)	[24]
PC4	NTG derived mutant of PAO1, *cheR*	[19]
TLPC1	PAO1 derivative, *tlpC*::*tet*	This study
TLPG1	PAO1 derivative, *tlpG*::*kan*	This study
TLPCG1	PAO1 derivative, *tlpC*::*tet tlpG*::*kan*	This study
Plasmids		
pBluescript II KS+	General cloning vector; Ap^r	Stratagene

pUC118	General cloning vector; Ap^r	[31]
pHSG396	General cloning vector; Cm^r	Takara Shuzo
pUC4K	pUC4 containing a 1.3-kb *kan* cartridge; Ap^r Km^r	Pharmacia
pUC118Tc	pUC118 containing a 1.3-kb *tet* cartridge from pBR322; Ap^r Tc^r	[16]
pCP19	Broad-host-range cosmid; Tc^r IncP	[34]
pMRP9-1	Broad-host-range GFP expression vector; Cb^r	[23]
pPT07.1	pCP19 derivative containing Che cluster 1 from *P. aeruginosa* PAO1; Tc^r	[19]
pTLPC01.2	pMRP9-1 derivative containing a 3.3-kb *tlpC* PCR product; Cb^r	This study
pTLPG01.2	pMRP9-1 derivative with a 2.1-kb *Sal*I fragment containing *tlpG* from *P. aeruginosa* PAO1; Cb^r	This study

[a] Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Cb, carbenicillin; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; GFP, green fluorescent protein.

[b] Gene ID numbers used in the *P. aeruginosa* genome sequencing project (http://www.pseudomonas. com/).

2.2. *Aerotaxis assay*

The chemotaxis well chamber method [8] was used to assess aerotaxis. *P. aeruginosa* strains were transformed by electroporation with plasmid pMRP9-1 [23] which contained the *gfp* gene under the control of the *lac* promoter. *P. aeruginosa* strains harboring pMRP9-1, grown overnight in 2 x YT medium with appropriate antibiotics, were inoculated into fresh 2 x YT medium (1% inoculum) and incubated at 37˚C with shaking for 4 h. Cells were harvested by centrifugation in a 1.5 ml Eppendorf tube for 4 min at 4000 x *g* at room temperature, washed twice with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2), and gently resuspended in 1 ml of the same buffer to a concentration of approximately 5×10^8 cells per ml. A 1 ml clear acrylic well (Chemotaxicell, Kurabo Co., Okayama, Japan) was used as an upper well. The bottom of the upper well was sealed by a 8 mm diameter polycarbonate filter with a uniform pore size of 8 μm. The upper well was placed in a 3 ml well of a 24-well microtitration plate (Microplate, Iwaki Co., Japan). This 3 ml well was used as a lower well. For each assay, 1.5 ml of cell suspension was added to the lower well. The upper well was filled with 0.75 ml of HEPES buffer. The assay was started by inserting the

upper well over the lower well allowing bacterial cells to cross the filter in response to an oxygen gradient. Bacterial cells that migrated to the upper well were automatically detected by measuring the green fluorescent protein (GFP) fluorescence intensity using a fluorescence spectrometer (ARVO 1420 Multilabel Counter, Wallac Co., Turku, Finland) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. All experimental measurements were performed at room temperature.

2. 3. *DNA manipulation and electroporation*

Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis [22]. PCR reactions were carried out using KOD plus DNA polymerase (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. *P. aeruginosa* was transformed by electroporation as described previously [18].

2.4. Construction of mutant genes/clusters

Deletion-insertion mutations were constructed as detailed below. Genetic organization of Che clusters and the locations of deletion-insertion mutations in *P. aeruginosa* strains are shown in Fig. 1. Oligonucleotides used for PCR are listed in Table 2.

Che cluster 1. A 2.0-kb *Cla*I-*Hpa*I fragment containing *fleN* and *fliA* from pPT07.1 [19], a 1.3-kb *Hin*cII-flanked *kan* gene cassette (conferring kanamycin resistance [Km^r]) from pUC4K , and a 2.0-kb *Hin*cII-*Hin*dIII fragment including open reading frames (ORFs) PA1465, PA1466, and PA1467 from pPT07.1 were ligated with the backbone of *Cla*I/*Hin*dIII-digested pHSG396 to obtain pPT07.20.

Cluster2. The isolation of the *cheR* mutant PC4 has been described previously [19].

Che cluster3. The deletion of the *pilHIJK* genes has been described previously [24]. A 0.7-kb DNA fragment containing a N- and C-terminal truncated copy of *pilJ* was amplified with the PCR primers pilJf and pilJr and

Table 2
Oligonucleotides used for PCR

Primer	Sequence (5' to 3')
Primers used for construction of ΔCHE3	
pilJf	TTTGCCTACCTCAACACCCAGTCG
pilJr	GCGAAACGCTGAAGATGTTGTTCG
chpBf	ATGGCTACGAGGTGGTGCTCAACG
cphBr	TCATGTTTCGACTCCTGTCGGCGC
Primers used for construction of ΔCHE4	
tlpFf	TCCCTCGGTGTTTCGTTACCATCC
cheY2r	TGCGCGTCACTTCGAGCAGTTTCG
Primers used for construction of ΔCHE5	
tlpMf	AAGATGTTGACGGTGGAAAAGGGC
cheW3r	AATGCCTCGACCCGCACCCATTGC
cheB3f	TCGAGGCAACTCGTCGGATCATGG
cheY3r	TTGGTCTCCAGCAACTGCTGCTGG
Primers used for construction of TLCP1	
tlpCf	GAAGGACTTCAACTCCTACGGCTCC
tlpCr	AAGCGACTGAAGCGTTTCCTCCACG

the PCR product was cloned into pUC118 to make pPT07.21. A 0.9-kb DNA fragment containing the 3' end of *chpB* amplified with the primers chpBf and chpBr was cloned into pUC118 to obtain pPT07.22. A 0.7-kb *Xba*I-*Eco*RI fragment from pPT07.21, a 1.3-kb *Eco*RI-flanked *kan* gene cassette from pUC4K, and a 0.9-kb *Eco*RI-*Sph*I fragment from pPT07.22 were ligated with the backbone of the *Xba*I/*Sph*I-digested pUC118 to obtain pPT07.23.

Che cluster 4. A 1.7-kb DNA fragment, which contained the entire *tlpF* gene and the 5' end of $cheY_2$, was amplified with the PCR primers tlpFf and cheY2r. The PCR product was cloned into pBluescriptII KS+ to make pPT07.24. A 1.0-kb *Sal*I-*Bam*HI chromosomal DNA fragment containing the 3' end of $cheB_2$ was cloned into pUC118 to obtain pPT07.25. A 1.7-kb *Xba*I-*Xho*I fragment from pPT07.24, a 1.3-kb *Sal*I-flanked *kan*

cassette from pUC4K, and a 1.0-kb *Sal*I-*Bam*HI fragment from pPT07.25 were ligated with the backbone of the *Xba*I/*Bam*HI-digested pUC118 to obtain pPT07.26.

Che cluster 5. A 1.1-kb DNA fragment containing the 3' end of *tlpM* and the 5' end of *cheW$_3$* was amplified with the primers tlpMf and cheW3r and the PCR product was cloned into pUC118 to make pPT07.27. A 1.4-kb DNA fragment containing the 3' end of *cheB$_3$* and the 5' end of *cheY$_3$* was amplified with the primers cheB3f and cheY3r and the PCR product was cloned into pUC118 to obtain pPT07.28. A 1.1-kb *Kpn*I-*Hin*cII fragment from pPT07.27, a 1.3-kb *Hin*cII fragment containing *kan*, and a 1.3-kb *Eco*RV-*Bam*HI fragment from pPT07.28 were ligated with the backbone of the *Kpn*I/*Bam*HI-digested pUC118 to obtain pPT07.29.

tlpC (*aer*). PCR was used to generate a fragment containing *tlpC*. The PCR product was cloned into pUC118 to give pTLPC01. pTLPC01 was partially digested with *Pst*I and ligated with a 1.3-kb *Pst*I-flanked *tet* gene cassette (conferring tetracycline resistance [Tc^r]) from pUC118Tc to obtain pTLPC01.1.

tlpG (*aer-2*). A 2.1-kb *Sal*I fragment containing *tlpG* was cloned into pUC118 to make pTLPG01. The *tlpG* gene was insertionally inactivated by cloning a 1.3-kb *Sal*I flanked *kan* gene cassette into the *Xho*I site in the *tlpG* ORF on pTLPG01 to give pTLPG01.1.

The mutations were transferred into the chromosome of PAO1 by allelic exchange as described previously [19]. The deletion-insertions were confirmed by Southern hybridization with a digoxigenin nonradioactive DNA labeling and detection kit (Roche Diagnostics).

3. Results and discussion

3.1. Che cluster 1 and cheR are required for aerotaxis.

The aerotactic responses of *P. aeruginosa* were assessed with the chemotaxis well chamber method [8]. When both of the upper and lower wells contained HEPES buffer alone, *P. aeruginosa* PAO1(pMRP9-1) moved from the lower to the upper well through the filter, responding to the gradient of oxygen. After the

gfp-tagged *P. aeruginosa* PAO1 cells were introduced to the lower well, the GFP fluorescence intensity in the upper well continuously increased (Fig. 2A). The *gfp*-tagged ∆pilHIJK (∆*pilH pilI pilJ pilK*), ∆CHE3 (∆*pilJ pilK pilL chpA chpB*), and ∆CHE5 (∆*cheW*$_3$ *cheR*$_3$ *cheW*$_4$ *cheA*$_3$ *cheB*$_3$) cells exhibited normal aerotactic responses, however, ∆CHE1 (∆*cheY cheZ cheA cheB motA*$_2$ *motB*$_2$ *cheW*) and PC4 (*cheR* mutant) had the impaired ability to respond to oxygen. Microscopic analysis showed that ∆CHE1 and PC4 were fully motile. ∆CHE1 and PC4 were also defective in chemotaxis toward peptone and phosphate (data not shown). pPT07.1 (carrying the Che cluster 1) restored the ability of ∆CHE1 to respond to oxygen (Fig. 2A). These results suggest that the mutation phenotypes were not due to polar effects of the cassette insertion and the Che cluster 1 were required for aerotaxis in *P. aeruginosa* PAO1. The *cheR* gene was shown to be essential for aerotaxis, suggesting that aerotaxis is the MCP-dependent chemotaxis [25].

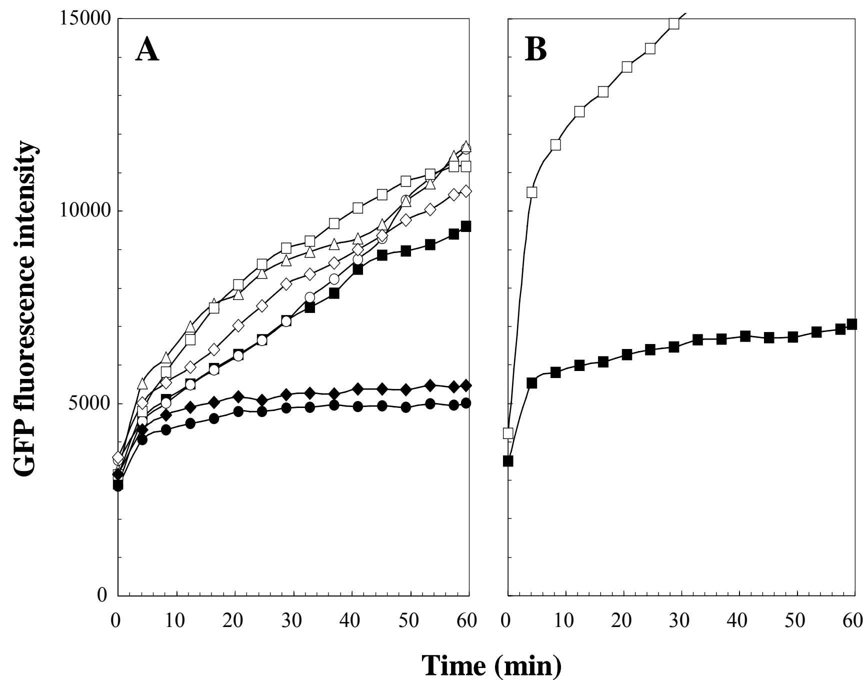

Fig. 2. Aerotactic responses by wild-type and mutant strains of *P. aeruginosa*. The changes in the GFP fluorescence intensity of the upper well were measured by a fluorescence spectrometer. (A) ❍, PAO1 (wild type); ℓ, ∆CHE1 (∆*cheY cheZ cheA cheB motA*$_2$ *motB*$_2$ *cheW*); ∆, ∆CHE3 (∆*pilJ pilK pilL chpA chpB*); ❑, ∆pilHIJK (∆*pilH pilI pilJ pilK*); , ∆CHE1(pPT07.1); ◊, ∆CHE5 (∆*cheW*$_3$ *cheR*$_3$ *cheW*$_4$ *cheA*$_3$ *cheB*$_3$); _, PC4 (*cheR* mutant). (B) ■, ∆CHE4 (∆*cheY*$_2$ *cheA*$_2$ *cheW*$_2$ *tlpG cheR*$_2$ *cheB*$_2$); ❑, ∆CHE4(pTLPG01.2).

3.2. TlpG is a chemotactic transducer for aerotaxis.

ΔCHE4 (Δ*cheY*$_2$ *cheA*$_2$ *cheW*$_2$ *tlpG cheR*$_2$ *cheB*$_2$) showed decreased aerotaxis (Fig. 2B). ΔCHE4 was fully motile. In contrast to ΔCHE1, ΔCHE4 exhibited positive chemotactic responses to peptone and phosphate (data not shown) and the Che cluster 4 contains the *mcp*-like gene, *tlpG*. These suggest the possibility that TlpG is a chemotactic transducer for aerotaxis and the mutation phenotype of ΔCHE4 is due to the deletion of the *tlpG* gene. To investigate the possibility, pTLPG01.2 (containing *tlpG*) was introduced into ΔCHE4 and the transformant was examined for aerotaxis. The introduction of pTLPG01.2 restored the ability of ΔCHE4 to respond to oxygen (Fig. 2B). To confirm that TlpG is a chemotactic transducer for aerotaxis, we inactivated the *tlpG* gene by inserting a *kan* cassette into the wild-type gene in the PAO1 genome. The single *tlpG* mutant, designated TLPG1, showed decreased aerotaxis (Fig. 3A). pTLPG01.2 complemented the mutation of TLPG1, indicating the absence of polar effects. ΔCHE4(pTLPG01.2) and TLPG1(pTLPG01.2) showed stronger aerotactic responses than PAO1 (Fig.2B and 3A). PAO1(pTLPG01.2) also showed stronger aerotaxis than PAO1 (Fig. 3A). These results indicate that the level of TlpG protein in the cell determines the strength of its positive aerotactic response.

3.3. Identification of TlpC as a second aerotaxis transducer

Although *tlpG*-deficient cells showed decreased aerotactic responses, aertaxis was not abolished in *tlpG*-deficient cells (Fig. 3A). This result suggests the existence of an additional transducer for aerotaxis. To identify an additional transducer for aerotaxis, a series of mutants that have deletion-insertion mutations in individual *mcp*-like genes in the PAO1 genome were constructed. PAO1 possesses 26 *mcp*-like genes [15-17]. We amplified 25 *mcp*-like genes other than *tlpG* by PCR using the sequence-specific primers, cloned into the vector plasmid pUC118, and disrupted individual genes by inserting a *tet* cassette into the wild-type genes in the PAO1 genome. After the introduction of pMRP9-1, each of mutants was tested for aerotaxis. Aerotaxis assays revealed that the *tlpC* (PA1561) mutant TLPC1 showed decreased aerotaxis (Fig. 3B). pTLPC01.2 carrying the *tlpC* gene complemented the mutation of TLPC1. TlpC overproduction also increased the aerotactic response (PAO1 [pTLPC01.2], Fig. 3B). We further constructed the double mutant TLPCG1 by

inserting a *tet* cassette into the wild-type *tlpC* gene in the TLPG1 genome. Aerotaxis was abolished in the *tlpC tlpG* double mutant TLPCG1 (Fig. 3C). Aerotaxis was partially restored in the *tlpC tlpG* double mutant when TlpC or TlpG was expressed from a plasmid. These results suggest that *P. aeruginosa* PAO1 possesses two aerotaxis transducers, TlpC and TlpG.

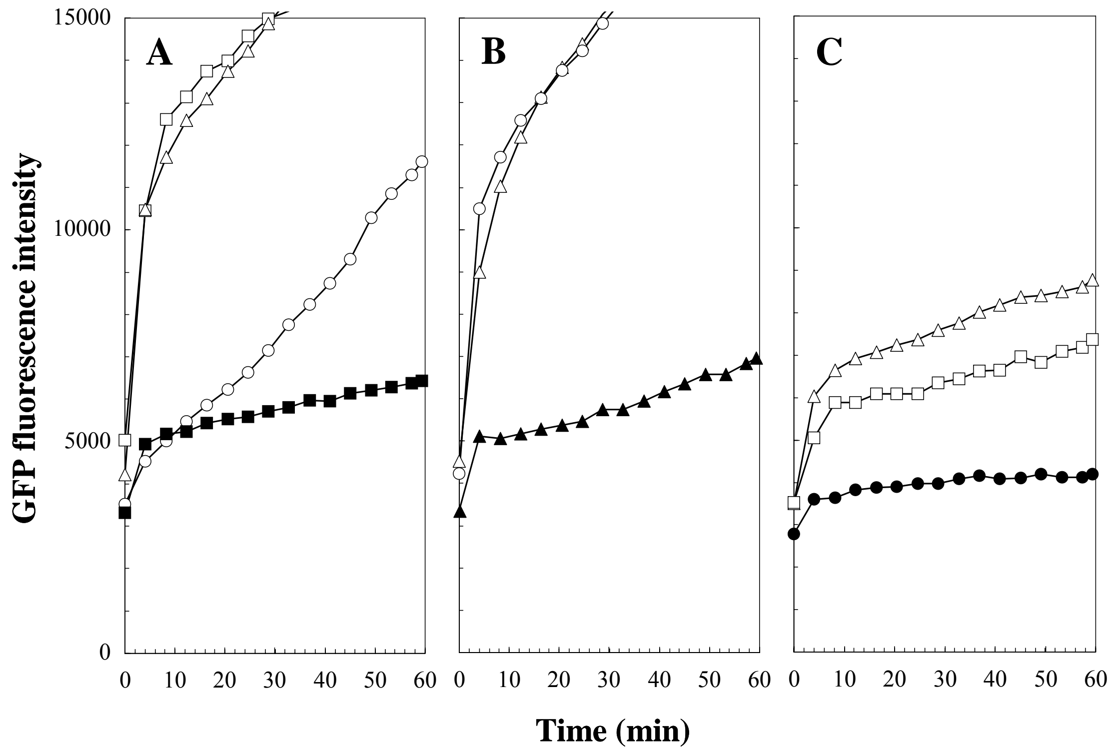

Fig. 3. Aerotactic responses by *P. aeruginosa* strains. (A) ∓, PAO1; □, TLGP1 (Δ*tlpG*); ∘, TLPG1(pTLPG01.2); Δ, PAO1(pTLPG01.2). (B) ⎽, TLPC1 (Δ*tlpC*); ∓, TLPC1(pTLPC01.2); Δ, PAO1(pTLPC01.2). (C) ℓ, TLPCG1 (Δ*tlpC tlpG*); Δ, TLPCG1(pTLPG01.2); ∘, TLPCG1(pTLPC01.2).

3.4. Sequence analysis of the tlpC and tlpG genes

The potential product of *tlpC* was 521-amino-acid TlpC. The *tlpC* gene product is 78 and 45%, respectively, identical to the *Pseudomonas putida* and *E. coli* Aer proteins, transducers involved in aerotaxis [11,12,26]. The N-terminal domain of the *tlpC* gene product (residues 21-106) contains a PAS motif, which is known to comprise binding pocket for prosthetic group [14]. Only one hydrophobic sequence (residues 146-191) was predicted in the TlpC protein. This hydrophobic sequence may serve to anchor TlpC to the cytoplasmic side of the inner membrane. TlpC residues 354 to 397 is 84% identical to the 44-amino-acid highly conserved domain

(HCD) of the *E. coli* chemotaxis transducer Tsr. MCPs from phylogenetically diverse bacteria have been shown to possess the HCD [27], which is likely to be important for the interaction between MCPs and CheW as well as CheA [28]. Thus the TlpC protein closely resembled the *P. putida* and *E. coli* Aer proteins and it was experimentally demonstrated that the *tlpC* gene is involved in aerotaxis. On this basis, the *tlpC* gene was renamed *aer*.

The putative product of the *tlpG* gene was a 679-residue protein (72.6 kDa) that had a predicted C-terminal segment (residues 481-524) with 89% identity to the HCD of the *E. coli* Tsr protein. In the C-terminal domain of TlpG, there are two potential methylation regions (residues 412-434 and 600-624) that have 87 and 72% identities to the K1 and R1 regions of *E. coli* Tsr [29], respectively. Thus, the C-terminal domain of TlpG has typical structural features of MCPs. A hydropathy plot of TlpG indicated that two hydrophobic membrane-spanning regions in the N-terminal domain of typical MCPs are missing in TlpG. TlpG may be a cytoplasmic protein and sense intracellular stimuli. Reverse position specific Blast search [30] against the database of the National Center for Biotechnology Information predicted the presence of a PAS motif in the N-terminal domain of TlpG (residues 174-216). TlpC and TlpG had 18% identity in the PAS domain. Since genetic analysis demonstrated the involvement of the *tlpG* gene in aerotaxis, *tlpG* was renamed *aer-2*. No significant homology other than the PAS motif was detected in the N-terminal domains between Aer and Aer-2.

Acknowledgements

We thank Matthew R. Parsek for providing the pMRP9-1 plasmid containing the *gfp* gene. This work was supported by Showa Shell Sekiyu Foundation for Promotion of Environmental Research and Kurita Research Foundation.

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