Journal of Bacteriology, Vol. 187, 1533-1535 (2005)

Expression of *Pseudomonas aeruginosa aer-2*, one of two aerotaxis transducer genes, is controlled by RpoS

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P. aeruginosa **possesses two aerotaxis transducers, Aer and Aer-2. A deletion-insetion mutant of the alternative sigma factor RpoS decreased aerotaxis. The *rpoS* mutation eliminated Aer-2-mediated aerotaxis, but not Aer-mediated aerotaxis. Transcriptional analysis revealed that *cheY*$_2$, *cheA*$_2$, *cheW*$_2$, and *aer-2* were expressed in an RpoS-dependent manner as a single transcript.**

An aerotactic response in *Pseudomonas aeruginosa* PAO1 (7) has previously been characterized as the movement of a cell towards oxygen (14). In *P. aeruginosa* PAO1, chemotaxis proteins such as CheA, CheB, CheR, CheW, and CheY are required and two methyl-accepting chemotaxis proteins (MCPs) Aer and Aer-2 function as independent sensor/transducers for aerotaxis (8). The aerotactic responses of *P. aeruginosa* cells were induced during the transition from exponential to stationary growth phase (9). In the previous study, we demonstrated that *aer* is transcriptionally regulated by the anaerobic regulator ANR (9). ANR is involved in the anaerobic induction of various enzymatic systems, including those required for arginine fermentation, cyanogenesis, and denitrification (1, 5, 6, 18). ANR activates target promoters by binding to ANR boxes, the consensus sequences shared by the ANR-dependent promoters (1, 6). The *aer* promoter contains two ANR boxes at -42.5 and -93.5 bp upstream of the transcriptional start site of *aer* and both of them are essential for expression of the *aer* gene (9). The *anr* mutation eliminated Aer-mediated aerotaxis, but not Aer-2-mediated aerotaxis, suggesting that *aer-2* expression is regulated by a factor other than ANR. In the present study, we report that the alternative sigma factor RpoS is required for Aer-2-mediated aerotaxis and the transcription of *aer-2* is dependent on RpoS.

The sigma factor RpoS is known to have a role in regulating the expression of stationary-phase genes in a wide range of bacteria, including *P. aeruginosa* (10, 15). It is possible that the RpoS is

involved in the stationary induction of Aer-2-mediated aerotaxis in *P. aeruginosa*. To assess the possibility that RpoS is involved in the stationary induction of Aer-2-mediated aerotaxis, the *rpoS* gene was disrupted by inserting a *tet* (conferring tetracycline resistance) cassette (17) into the wild-type gene in the *P. aeruginosa* PAO1 genome as described previously (11). The resulting *rpoS* mutant, designated PAO-CH1, was fully motile and grew as well as the parent strain PAO1. PAO-CH1 was examined for the ability to exhibit aerotaxis.

Aerotactic responses of *P. aeruginosa* were assessed with the chemotaxis well chamber method (14). In this method, 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., Tokyo, Japan). This 3 ml well was used as a lower well. *P. aeruginosa* PAO1 was transformed with the green fluorescent protein (GFP) expression vector pMRP9-1 (13). 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. 1). Stationary-phase cells of the *rpoS* mutant PAO-CH1 (pMRP9-1) exhibited decreased, but significant aerotaxis. The entire *rpoS* gene (15) was cloned into pMRP9-1 to construct pCSH9-11. Plasmid pCSH9-11 complemented the mutation of PAO-CH1 (Fig. 1), showing that the mutation phenotype was not due to polar effects. The intensity of aerotaxis by PAO-CH1 was as strong as those of the *aer* or *aer-2* single mutants (8) (Fig. 1). These results suggest the possibility that RpoS regulates only one of the aerotaxis transducer genes. To confirm this possibility, we constructed the *aer rpoS* and *aer-2 rpoS* double mutants by inserting a *kan* (conferring kanamycin resistance) cassette into the wild-type *aer* and *aer-2* genes in the PAO-CH1 genome, respectively. The *aer rpoS* and *aer-2 rpoS* double mutants were designated PAO-CH2 and PAO-CH3, respectively. Aerotaxis assays revealed that PAO-CH2 failed to exhibit aerotaxis, whereas PAO-CH3 showed the same level of aerotactic reponses as the *aer-2* single mutant (Fig. 1). These results demonstrated that Aer-2-mediated aerotaxis, but not Aer-mediated aerotaxis, requires RpoS.

The *aer-2* gene is associated with a *mcp*-like gene (*tlpF*) and a complete set of chemotaxis-like genes (*cheY*$_2$, *cheA*$_2$, *cheW*$_2$, *cheR*$_2$, and *cheB*$_2$) encoding homologues of CheY, CheA, CheW, CheR, and CheB (8) (Fig. 2). These genes are juxtaposed and have the same transcriptional polarity. *tlpF*, *cheY*$_2$, *cheA*$_2$, and *cheW*$_2$ are located upstream of *aer-2*. They are overlapped or separated by short intergenic regions (27-197 bp), suggesting that these genes are expressed as a single transcript. To locate the promoter of the transcript containing *aer-2*, we constructed promoter fusions in the broad-host range transcriptional fusion vector pQF50 (3). Regions upstream of *aer-2* were isolated and inserted individually upstream from the promoterless *lacZ* gene in pQF50 (Fig. 2). Each of five constructs was transformed into PAO1 and PAO-CH1. β-Galactosidase activities were then measured in stationary-phase cells of transformant strains of *P. aeruginosa*. β-Galactosidase activities were determined as described by Miller (12), with the modification that enzymatic

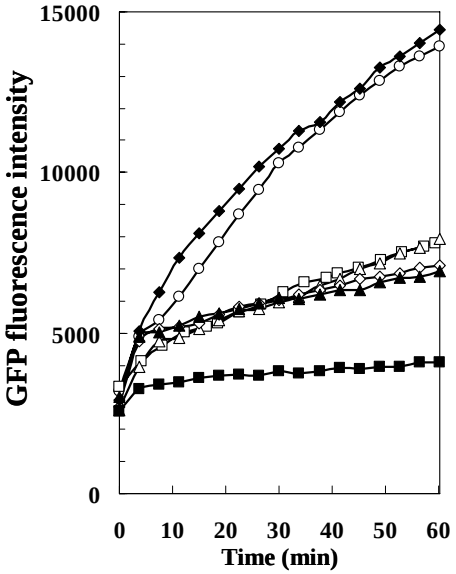

Fig. 1. Aerotactic responses by wild-type and mutant strains of *P. aeruginosa*. The changes in the GFP fluorescence intensity of the upper well were measured by a fluorescence spectrophotometer. ❍, PAO1 (pMRP9-1) (wild type); △, TLPC01 (pMRP9-1) (*aer* mutant) (8); ❑, TLPG01 (pMRP9-1) (*aer-2* mutant) (8); ◇, PAO-CH1 (pMRP9-1) (*rpoS* mutant); ▲, PAO-CH3 (pMRP9-1) (*aer-2 rpoS* mutant); ■, PAO-CH2 (pMRP9-1) (*aer rpoS* mutant); ◆, PAO-CH1 (pCSH9-11).

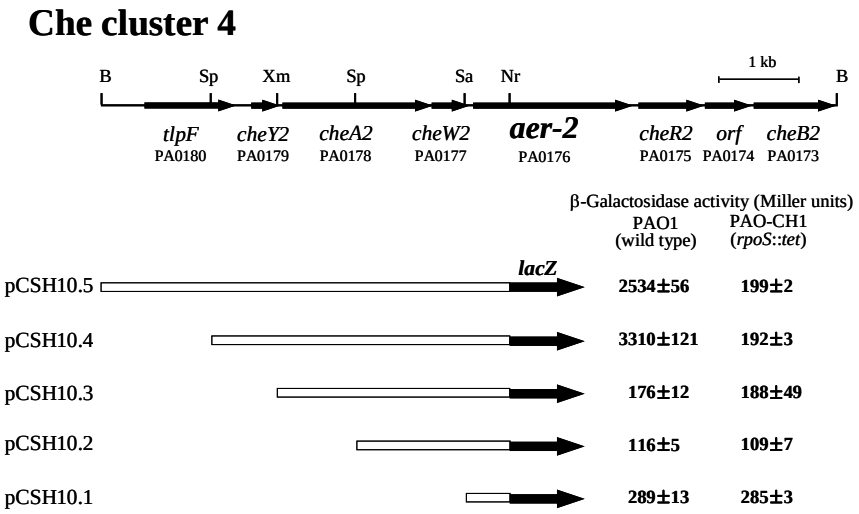

Fig. 2. Physical map of the Che cluster 4 of *P. aeruginosa* PAO1 (8) and regions cloned in the promoter probe vector pQF50. Specific restriction sites used to isolate each fragment are shown on the map. Restriction sites: B, *Bam*HI; Nr, *Nru*I; Sa, *Sau*3AI; Sp, *Sph*I; Xm, *Xmn*I. The locations and orientations of *tlpF*, *cheY*$_2$, *cheA*$_2$, *cheW*$_2$, *aer-2*, *cheR*$_2$, PA0174, and *cheB*$_2$ are indicated by horizontal arrows. Open bars are *P. aeruginosa* chromosomal DNA fragments subcloned into pQF50. The *lacZ* gene is shown by the black arrow. β-Galactosidase activities were determined in *P. aeruginosa* wild-type PAO1 and its *rpoS* mutant PAO-CH1 containing the *aer-2*::*lacZ* transcriptional fusion plasmids shown. β-Galactosidase activity is shown along with the standard deviation (mean of four independent experiments).

reaction was carried out at 37˚C. High levels of β-galactosidase activities were detected with PAO1(pCSH10.5 [carrying *tlpF-cheY*$_2$*A*$_2$*W*$_2$*-aer-2*::*lacZ*]) and PAO1(pCSH10.4 [carrying *cheY*$_2$*A*$_2$*W*$_2$*-aer-2*::*lacZ*]) (Fig. 2). In PAO1 harboring pCSH10.3 (carrying *cheA*$_2$*W*$_2$*-aer-2*::*lacZ*), pCSH10.2 (carrying *cheW*$_2$*-aer-2*::*lacZ*), and pCSH10.1 (carrying *aer-2*::*lacZ*), β-galactosidase levels were about 150 units, which was similar to the basal levels seen in PAO1 harboring the control plasmid pQF50. These results suggest that *cheY*$_2$, *cheA*$_2$, *cheW*$_2$, and *aer-2* are expressed as a single transcript and the transcript starts from the region upstream of *cheY*$_2$. The *lacZ* fusion pCSH10.4 gave a basal level of β-galactosidase activity in PAO-CH1, demonstrating that RpoS is required for the transcription of the *cheY*$_2$*A*$_2$*W*$_2$*-aer-2* genes.

To identify the 5' end of the mRNA containing *cheY*$_2$*A*$_2$*W*$_2$*-are-2*, we carried out rapid amplification of cDNA ends (RACE) as described previously (4, 9). The *cheY*$_2$ sequence specific primer Y2SPI (5'- GCGTCACTCGAGCAGTTTC-3') was used for the reversetranscription of total RNA from PAO1. The nested PCR using the forward primers T17ADP (5'-GAGTCGACTCGAGAATTCTTTTTTTTTTTTTTTT-3') and ADP (5'-GAGTCGACTCGAGAATTC-3') and the *cheY*$_2$ sequence specific primers Y2SPII (5'-TTCATCGCGTCGCTCGATTC-3') and Y2SPIII (5'-ATTCGGTGGTCAGCATGAGG-3') enabled amplification of a RACE product (data not shown) that was subsequently isolated and subcloned into pUC118 (16). DNA sequencing indicated that the 5' ends were located at an A nucleotide -101 and a G nucleotide -100 (relative to the *cheY*$_2$ start codon) (Fig. 3). A potential RpoS -10 region (CTTTACT) was located in positions -13 to -7 upstream of the transcription start point (the A nucleotide) (Fig. 3), with six of the seven bases being identical to the consensus sequence (CTATACT) (2).

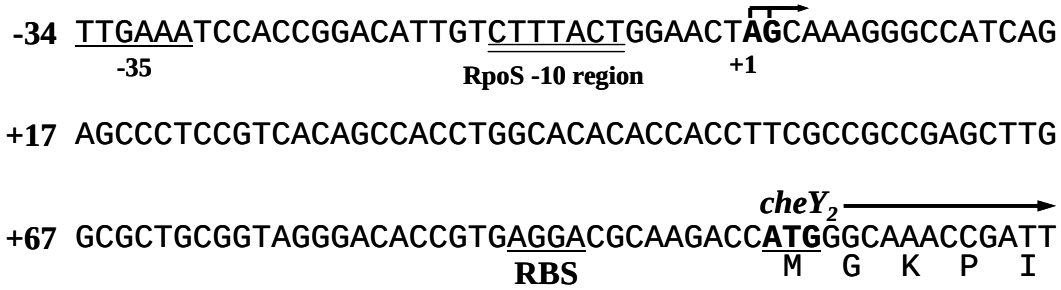

Fig. 3. Promoter region of *P. aeruginosa cheY*$_2$. The transcriptional start sites, which were determined by 5'-RACE and DNA sequencing, are indicated by the angled arrow. Numbering is relative to the transcriptional start site (the A nucleotide). The putative translation start site, ribosome-binding site (RBS), and -35 promoter sequence are underlined. Double underlining indicates a putative RpoS -10 region.

In summary, *P. aeruginosa* possesses two aerotaxis transducers, Aer and Aer-2. *aer* expression

is dependent on the anaerobic regulator ANR, which is converted to its active form under low oxygen supply. *aer-2* is transcribed together with *cheY*$_2$*A*$_2$*W*$_2$ (and probably with *cheR*$_2$*B*$_2$). The stationary-phase sigma factor RpoS is required for transcription of the operon.

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