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Original

Identification of the transcriptional regulatory gene of histidase and urocanase in *Pseudomonas aeruginosa*

—— Regulation of histidase and urocanase expression ——

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Abstract

Pseudomonas aeruginosa is a typical opportunistic bacterial pathogen that can grow under poor nutrient conditions. Histidine utilization as a sole source of carbon and nitrogen is a distinctive feature of *P. aeruginosa*, which decompose and utilize a variety of nutrients and complex compounds using various catabolite pathways. When wild-type *P. aeruginosa* PAO1 cells were cultured in minimal medium P (MMP) containing 20 mM histidine, synthesis of histidase and urocanase was detected in crude extract at 3186±118 (U/mg-proteins) and 243±3 (10^{-3} U/mg-proteins), respectively. It was determined that the synthesis of these enzymes was induced by histidine because limited enzyme synthesis was observed in MMP containing 20 mM of glutamate. In contrast, PAO4399, the spontaneous mutant of PAO1, can synthesize histidase and urocanase sufficiently under the nutrient conditions of MMP containing histidine or glutamate. A nucleotide sequence analysis of PAO4399 showed a C to G transition at nt 441 of the PA5105 gene, with this mutation causing an amino acid change of the tyrosine codon (TAC) Tyr 147 to stop codon (TAG). In the case of PAO4816, the knockout mutant strain of PAO1 with an inserted gentamicin (Gm)-cassette in the PA5105 gene, the cells grown in MMP containing 20 mM of histidine or glutamate synthesized histidase and urocanase constitutively, in the same manner as PAO4399. The PA5105 gene product is highly homologous with the *hutC* gene product of *Pseudomonas putida*, which regulates histidase and urocanase gene expression. These findings support the role of the PA5105 gene of *P. aeruginosa* PAO1 as a repressor-type *hutC* gene that regulates histidase and urocanase synthesis under histidine-dependent nutrient conditions.

Key words: *Pseudomonas aeruginosa*, repressor, histidase, urocanase

INTRODUCTION

Pseudomonas aeruginosa is a prevalent opportunistic human pathogen that causes acute pneumonia, cystic fibrosis, and septicemia in immunocompromised individuals (Govan & Deretic, 1996). It is known that pseudomonads have various natural and acquired resistance systems to antibiotics, and that some of these systems involve decomposition of complex compounds such as hydrolysis by β-lactamase (Aloush *et al.*, 2006). *P. aeruginosa* lives in soil and aqueous environments, including those found in the nursing-station sinks and bathrooms of medical facilities, where it survives due to environmental adaptations that allow it to utilize a variety of nutrients through many kinds of catabolic enzymes and highly developed gene expression and regulation systems (Nishijyo *et al.*, 2001). Nutritionally versatile pseudomonads effectively utilize various nitrogenated compounds, including histidine, as sources of carbon, nitrogen, and energy.

The histidine catabolic pathway of Pseudomonas was first established in *Pseudomonas fluorescens*, followed by *Pseudomonas putida* and *P. aeruginosa* (Lessie & Neidhardt, 1967). The genes for the pathway enzymes were then identified and characterized in *P. putida* (Hu & Phillips, 1988). The catabolism proceeds in

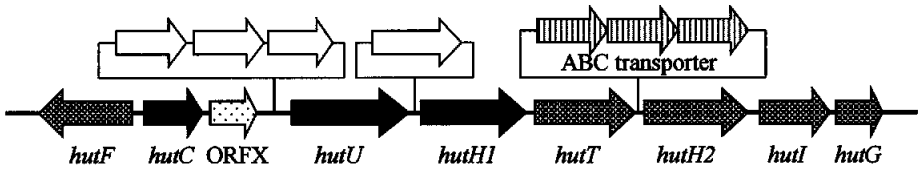

Fig. 1 Organization of putative *hut* genes of *P. aeruginosa* PAO1

five enzymatic reactions (the product of *hutHUIFG*) that initially deaminate histidine to urocanate by histidase (Hernandez & Phillips, 1993). Expression of the histidine catabolic enzymes requires the presence of histidine (or its degradation intermediate urocanate), and HutC behaves as a repressor-type transcriptional regulator of the *hut* operon (Hu & Phillips, 1988). *P. aeruginosa* also has a genomic region containing a cluster of histidine catabolism *hut* genes (Fig. 1), but gene arrangement of this region is complex compared with that in other *Pseudomonas* spp (Stover *et al.*, 2000). In *P. aeruginosa* PAO1, the PA5105 gene in the *hut* gene cluster is annotated as *hutC* inferred from sequence and structural similarity (http: //www.pseudomonas.com/getAnnotation.do?locusID=PA5105), but there is currently no experimental evidence available. In the present study, we investigated histidase and urocanase activities, and analyzed the PA5105 gene using wild-type PAO1 and histidine utilization mutant strains to identify the role of the PA5105 gene product.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. PAO1 is a wild-type standard strain of *P. aeruginosa*, and PAO4399 is a histidine-assimilation spontaneous mutant of PAO1. *Escherichia coli* and *P. aeruginosa* strains were cultured in Luria-Bertani (LB) and nutrient yeast broth (NYB), respectively, and supplemented with antibiotics when appropriate (Haas *et al.*, 1977; Sambrook *et al.*, 1989). For enzyme assays, *P. aeruginosa* strains were grown in minimal medium P (MMP) with supplements of carbon and nitrogen sources at 20 mM as described previously (Haas *et al.*, 1977).

DNA techniques

DNA purification, restriction enzyme analysis, and other DNA manipulations were conducted as previously described (Itoh, 1997; Sambrook *et al.*, 1989). Polymerase chain reaction (PCR) was performed with KOD-plus DNA polymerase (Toyobo Biochemicals, Japan) under the reaction conditions recommended by the supplier, and nucleotides were sequenced using an ABI3130 DNA sequencer

Table 1 Strains and plasmids used in this study

Strains & plasmids	Relevant characteristics	Reference or source
Strains		
P. aeruginosa		
PAO1	wild type	Stover CV *et al.* 2000
PAO4399	*spontaneous hutC mutant*	This study
PAO4816	*hutC*:: *FRT*-Gm	This study
E. coli		
DH5α	*F⁻/endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1 recA1 gyrA relA1 Δ(lacIZYA-argF) U169 deoR (F80dlacΔ(lacZ)M15)*	Bethesda Research Laboratories
HB101	*supE44 hsdS20* (r_B^-,m_B^+) *recA13 ara-14 proA2 lacY galK2 rpsL20 xzl-5 mtl-1*	Sambrook *et al.* 1989
Plasmids		
pEX18Ap	Ap^r, ColE1 replicon, *oriT sacB*	Hoang *et al.* 1998
pPS858	Ap^r, Gm^r, ColE1 replicon, carring a *FRT*-Gm cassette	Hoang *et al.* 1998
pRK2013	Km^r, ColE1 replicon, *tra* (RK2)	Comai *et al.* 1983
pUC118	Ap^r, ColE1 replicon	Vieira *et al.* 1987

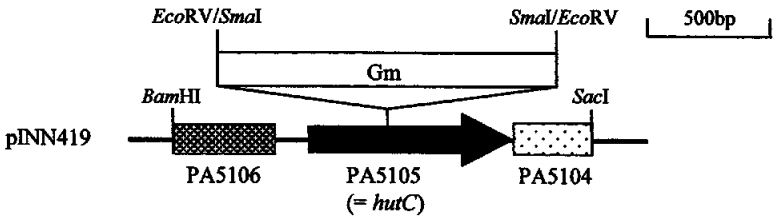

Fig. 2 Gene organization of the plasmid used in the PA5105 (*hutC*) knockout mutant strain

and a Big-Dye Terminator Sequencing Kit (Applied Biosystems, USA), as described previously (Nakada & Itoh, 2002).

Enzyme assays

P. aeruginosa strains were grown to an optical absorbance of 0.5 at 600 nm in MMP containing histidine or glutamate at 20 mM and harvested by centrifugation. Cell extracts were prepared by passing cells through a French pressure cell at 8,000 lb/in^2, and cell debris was removed after centrifugation at 15,000 g for 30 min. The supernatant was used as a crude extract and kept on ice until assays were performed. The histidase was measured by spectrophotometer at 277 nm using the method described by Rechler and Tabor (1971). Urocanase activity assay was performed as described by Lessie and Neidhardt (1967), and the amount of urocanate was also determined spectrophotometrically at 277 nm. Protein concentration was analyzed by the Bradford method using a Quick Start Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

PA5105 gene nucleotide sequencing of PAO4399

To construct a PA5105 gene sequencing plasmid of PAO4399, we amplified the PA5105 gene coding region by PCR using PAO4399 chromosomal DNA as a template and oligonucleotide primers designed to add *Nhe*I and *Xho*I sites (underlined) at the ends: 5'-TCATATGACGTCCTCTTCTTCCGATCGTT-3' (corresponding to nt 1 to 25 of PA5105) and 5'-GCTCGAGTGAGCTGAAACGTCCTTCCA-GA-3' (complementary to nt 728 to 750 of PA 5105). The amplified DNA fragment was cloned into plasmid pUC118 (Vieira & Messing, 1987) at the *Hinc*II site to verify the PA5105 gene nucleotides by sequencing. Sequence assembly was performed using ATGC version 5.0 (Genetyx Corporation, Japan).

Construction of PA5105 gene knockout mutant strain

A knockout mutant strain of the PA5105 gene was constructed by gene replacement according to the procedure described by Hoang *et al.* (1998). We amplified the PA5105 gene coding region by PCR using PAO1 chromosomal DNA as a template and oligonucleotide primers designed to add *Bam*HI and *Sac*I sites (underlined) at the ends: 5'-TTGGATCCGGGCAGCAGGGTCAGGCCGAT-ACCT-3' (corresponding to nt −568 to −544 of PA5105) and 5'-AAGAGCTCGCACAGG-CAGTCGTGGGCAGCCAGTT-3' (complementary to nt 479 to 504 of PA5104). The 1.8 kbp *Bam*HI-*Sac*I fragment containing the PA5105 gene was cloned into the conjugation vector pEX18Ap at the same site, resulting in pINN 418. A gentamicin (Gm)-resistant cassette from pPS858 was inserted as a *Sma*I fragment into the *Eco*RV site of PA5105 on pINN418. The resulting plasmid pINN419 was then conjugated into strain PAO1 via helper *E. coli* HB101/pRK2013 (Comai *et al.*, 1983; Fig. 2). The PA5105:: Gm mutant strain was selected on MMP agar containing 50 μg ml^{-1} Gm and 20 mM glutamate, and then on LB agar containing 5% sucrose, which allows the growth of clones lost during the second crossover of the plasmid sequence (*sacB*$^+$) integrated into the chromosome (Hoang *et al.*, 1998).

RESULTS

Histidase and urocanase synthesis in PAO1 and PAO4399

In the case of wild-type PAO1, when cells were cultured in MMP containing 20 mM of histidine as a carbon and nitrogen source, histidase synthesis was increased to 3186 ± 118 U/mg-protein based on analysis of crude extract. Under the growth conditions of MMP containing 20 mM of glutamate, little histidase

Table 2 Histidase and urocanase activity of PAO1, PAO4399, and PAO4816 under different growth conditions

Enzymes	C and N sources in growth medium	PAO1 (wild type)	PAO4399 (spontaneous mutant)	PAO4816 (*hutC*:: Gm)
Histidase	Glutamate	17±1	2796±115	2844±122
	Histidine	3186±118	3208±130	3308±161
Urocanase	Glutamate	1±0	187±15	199±19
	Histidine	243±3	236±14	226±12

*Cell extracts were prepared from cells grown exponentially in MMP containing 20 mM of the histidine or glutamate, and were used for enzyme assays.
†Histidase activity is presented as U/mg-protein of crude extract, and urocanase activity is presented as 10^{-3} U/mg-protein of crude extract.

synthesis was observed (Table 2). In contrast, PAO4399 cells grown in MMP containing 20 mM of glutamate synthesized histidase up to a level of 87% of the histidine presence. Urocanase, the secondary enzyme of the histidine degradation pathway, demonstrated similar behavior under the nutritional conditions of MMP containing 20 mM of histidine or glutamate (Table 2).

Sequence analyses of the PAO4399 gene PA5105

PA5105 gene nucleotide sequence analysis showed a C to G transition at nt 441, with this mutation causing an amino acid change of the tyrosine codon (TAC) Tyr 147 to stop codon (TAG).

Histidase and urocanase synthesis in PA5105 knockout mutant strain

The knockout mutant strain of PA5105 was constructed by gene replacement as described above, yielding the PAO4816 strain. When PAO4816 cells were cultured in MMP containing 20 mM of histidine or glutamate, induction of histidase and urocanase synthesis was observed as well as PAO4399 (Table 2).

DISCUSSION

Histidine catabolism proceeds in five enzymatic reactions in pseudomonads. Histidase (the product of *hutH*) initially deaminates histidine to yield urocanate, which is then converted to 4-imidazolone-5-propionate by urocanase (*hutU*). In the third reaction, imidazolone propionase (*hutI*) generates N-formimino-L-glutamate from 4-imidazolone-5-propionate. N-Formiminoglutamate deiminase (*hutF*) degrades the diverging intermediate into ammonia and N-formyl-L-glutamate, which is successively hydrolyzed by N-formylglutamate deformylase (*hutG*) to L-glutamate and formate (Consevage *et al.*, 1985; Hu *et al.*, 1987). The *hutHUIFG* system of histidine utilization gene identification and the transcriptional regulation of the repressor-type GltR protein family of HutC in *hutHUIG* expression have been investigated in *P. putida* (Hu & Phillips, 1988). In addition, this organism has two other genes, *hutT* and ORFX (*hutD*), that are also conserved in the *hut* gene clusters of *P. aeruginosa* (Fig. 1). The *hutT* gene encodes a hypothetical 50 kDa histidine transporter, and the ORFX has no sequence feature suggestive of its function (Zhang *et al.*, 2006). Additionally, the *hut* gene cluster in *P. aeruginosa* carries three sets of gene insertions containing putative ABC transporters. Moreover, there are two *hutH* like genes (*hutH 1, hutH2*), which probably duplicates the *hutH* gene. In *P. aeruginosa*, the *hut* gene cluster is so complicated that the genes that participate in histidine degradation and gene expression regulations are not well understood.

In the current study, we found a spontaneous mutant of PAO1, PAO4399, that synthesizes histidase and urocanase under histidine-independent conditions. Nucleotide-sequencing analysis showed that PAO4399 has a C to G transition at nt 441 of the PA5105 gene, with the mutation causing an amino acid change of Tyr-147 to a stop codon. This shows that the constitutive expression of histidase and urocanase under the nutrient conditions of MMP containing glutamate was caused by the incomplete PA5105 gene encoding protein production. Results of an additional study of histidase and urocanase synthesis in PAO 4816, the knockout mutant strain of PA5105 gene, support this finding. Finally, the PA 5105 gene product is 94% similar to the *hutC* gene product of *P. putida* (http: //www.pseudomonas.com/getAnnotation.do?locusID =PA5105). These findings support the conclusion that the PA5105 gene of *P. aeruginosa* PAO1 acts as a repressor-type *hutC* gene that regulates histidase and urocanase synthesis under histidine-dependent nutrient conditions.

With respect to histidine degradation gene expression in *P. aeruginosa*, there is a report that the *hutU* promoter-*lacZ* translational fusion plasmid containing PAO1 induced expression of β-galactosidase in the presence of histidine (Wei & Chung-Dar, 2007). In the current experiment, histidase and urocanase synthesis were synchronized so that there are two possibilities to explain it. One is that *hutU* and *hutH* have a promoter serially and HutC could repress both promoters; the other is that the *hutU*-PA5099-*hutH* gene induces an operon and HutC represses the promoter. Moreover, histidine utilization is also influenced by the CbrAB and NtrBC two-component regulatory systems, which control the utilization of multiple carbon and nitrogen sources (Nishijyo *et al.*, 2001; Wei & Chung-Dar, 2007). For the particulars of histidine utilization gene regulation, further analysis of the relationship between HutC and these two-component regulatory systems is needed.

In *P. aeruginosa*, annotated histidine utilization gene disposition is complicated compared with that in *P. putida*. Therefore, full identification of histidine utilization genes and the regulation of their expression in *P. aeruginosa* are recommended.

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