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Genetic Analysis of the Histidine Utilization (*hut*) Genes in *Pseudomonas fluorescens* SBW25

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ABSTRACT

The histidine utilization (*hut*) locus of *Pseudomonas fluorescens* SBW25 confers the ability to utilize histidine as a sole carbon and nitrogen source. Genetic analysis using a combination of site-directed mutagenesis and chromosomally integrated *lacZ* fusions showed the *hut* locus to be composed of 13 genes organized in 3 transcriptional units: *hutF*, *hutCD*, and 10 genes from *hutU* to *hutG* (which includes 2 copies of *hutH*, 1 of which is nonfunctional). Inactivation of *hutF* eliminated the ability to grow on histidine, indicating that SBW25 degrades histidine by the five-step enzymatic pathway. The 3 *hut* operons are negatively regulated by the HutC repressor with urocanate (the first intermediate of the histidine degradation pathway) as the physiological inducer. 5′-RACE analysis of transcriptional start sites revealed involvement of both σ^{54} (for the *hutU–G* operon) and σ^{70} (for *hutF*); the involvement of σ^{54} was experimentally demonstrated. CbrB (an enhancer binding protein for σ^{54} recruitment) was required for bacterial growth on histidine, indicating positive control of *hut* gene expression by CbrB. Recognition that a gene (named *hutD*) encoding a widely distributed conserved hypothetical protein is transcribed along with *hutC* led to analysis of its role. Mutational and gene fusion studies showed that HutD functions independently of HutC. Growth and fitness assays in laboratory media and on sugar beet seedlings suggest that HutD acts as a governor that sets an upper bound to the level of *hut* activity.

PSEUDOMONAS fluorescens strain SBW25 is a common saprophytic bacterium that activates expression of a suite of amino acid uptake and degradation pathways when growing in the plant environment (Rainey 1999; Gal *et al.* 2003). The histidine uptake and utilization pathway (the *hut* locus) confers on SBW25 the ability to utilize histidine as a sole source of carbon, nitrogen, and energy (Zhang *et al.* 2006).

In bacteria, catabolism of histidine occurs via either a four or a five-step enzymatic pathway (Coote and Hassall 1973a; Magasanik 1978). The first three steps, from histidine to urocanate, to imidazolone propionate (IPA), to formiminoglutamate (FIGLU), are catalyzed by the gene products of *hutH*, *hutU*, and *hutI*, respectively, and are common to both the four-step and the five-step pathways (outlined in Figure 1A). Breakdown of FIGLU differs among organisms. Enteric bacteria, *e.g.*, *Salmonella typhimurium* and *Klebsiella aerogenes* (Magasanik 1978), and the gram-positive bacterium *Bacillus subtilis* (Chasin and Magasanik 1968) hydrolyze FIGLU directly to form glutamate and formamide. However, *P. putida* (Hu *et al.* 1987) and *Streptomyces coelicolor* (Kendrick and Wheelis 1982) are known to employ two enzymes (FIGLU iminohydrolase encoded by *hutF* and formylglutamase encoded by *hutG*) to convert FIGLU into glutamate plus formate with formylglutamate (FG) as an intermediate. Notably, no ATP is generated in this process; thus the energy (and most building blocks) required for growth on histidine must be derived from further degradation of glutamate.

Regulation of *hut* expression is complex and not fully understood. In gram-negative bacteria, *hut* is negatively regulated by the product of *hutC* (Magasanik 1978; Allison and Phillips 1990). Repression by HutC is relieved by urocanate, the first intermediate of the histidine degradation pathway (Lessie and Neidhardt 1967; Newell and Lessie 1970), which interacts with the HutC repressor (Hu *et al.* 1989). In enteric bacteria, expression of *hut* requires derepression (of HutC) and also requires activation by additional positive regulation factors. When histidine is utilized as a source of carbon, *hut* transcription is activated by catabolite-activating protein (CAP) charged with cAMP. Thus, like the *lac* operon, expression is subject to control by catabolite repression exerted by glucose (Magasanik 1978). However, when histidine is a nitrogen source, *hut* expression is activated by the nitrogen assimilation control protein (NAC), whose transcription is controlled by the NtrBC two-component system in response to nitrogen starvation (Pomposiello *et al.* 1998).

Little is known about positive regulation of *hut* in Pseudomonas species, although succinate-provoked carbon catabolite repression has been observed (Lessie and Neidhardt 1967; Coote and Hassall 1973b;

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Phillips and Mulfinger 1981), an effect thought to be attributable to the inhibitory effects of succinate on urocanase (Hug *et al.* 1968). Recently, Nishijyo *et al.* (2001) showed that expression of the *hut* enzymes in *P. aeruginosa* requires the two-component system CbrAB, but just how CbrAB regulates *hut* transcription is unclear.

Here we report a genetic analysis of the *hut* locus from *P. fluorescens* SBW25. The study builds upon a substantive body of mainly enzymatic data acquired during the 1980s by Phillips and co-workers who focused on *P. putida* ATCC 12633 (Hu *et al.* 1987, 1989; Hu and Phillips 1988; Allison and Phillips 1990). Our work began with the unannotated whole-genome sequence of SBW25. Initially on the basis of the analysis of this sequence, but confirmed using genetics, we show that the *hut* locus is composed of 13 genes organized in 3 transcriptional units. We reveal the identity of the gene encoding FIGLU iminohydrolase (*hutF*), show that one copy of *hutH* is nonfunctional, and confirm negative regulation via HutC (and the role of urocanate as the specific inducer). In addition, we identify positive regulators (σ^{54} and the enhancer-binding protein CbrB) and attribute a phenotype to the widely distributed and highly conserved protein encoded by *pflu0360* (*hutD*).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions: *Escherichia coli* DH5αλ_{pir} was used for gene cloning and conjugative transfer into *P. fluorescens*. *P. fluorescens* strains and plasmids are listed in Table 1. Pseudomonas and *E. coli* strains are routinely grown in Luria–Bertani medium (LB) at 28° and 37°, respectively (Sambrook *et al.* 1989). Where indicated, Pseudomonas strains were also cultivated in minimal-M9 medium (Sambrook *et al.* 1989) supplemented with glucose (0.4% or 22.2 mm) and NH_4Cl (1 mg ml^{-1} or 18.7 mm). When histidine or urocanate were used as sole carbon and nitrogen sources, they replaced the glucose and ammonia of the M9 medium and were added at a final concentration of 15 mm. When necessary, antibiotics were included at the following concentrations: tetracycline (Tc), 10 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; spectinomycin (Sp), 100 μg ml^{-1}; nitrofurantoin (Nf), 100 μg ml^{-1}. M9 plates containing half-strength cetrimide, fucidin, and cephalosporin (CFC) supplement from Oxoid were used to select for *P. fluorescens* recovered from the sugar beet seedlings. Glutamine (1 mm) was added to minimal-M9 medium to support growth of the *rpoN* mutant (PBR808).

Growth kinetics of *P. fluorescens* SBW25 and the derived mutant strains were determined in microtiter plates using a VersaMax microtiter plate reader with SOFTmax PRO software (Molecular Devices). To ensure that all bacteria were physiologically equivalent, strains were inoculated from cells stored in a −80° freezer. They were first grown in LB broth (24 hr) and then subcultured once in M9 broth (24 hr) before use in assay conditions. Absorbance at the wavelength of 450 nm was determined every 5 min over a period of 48 hr.

Mutational analysis and complementation: Site-directed and insertional mutagenesis was performed using standard DNA manipulation techniques (Sambrook *et al.* 1989). Gene mutations were achieved by SOE–PCR (splicing by overlapping extension using the polymerase chain reaction; Horton *et al.* 1989) in conjunction with a two-step allelic-exchange strategy using the suicide-integration vector pUIC3 (Rainey 1999). Details of oligonucleotide primers are available on request. All PCR-generated fragments were checked by sequencing prior to exchange into the chromosome.

Cycloserine enrichment was used to enrich for strains that had lost the chromosomally integrated pUIC3-based vector. Strains were grown overnight in 20 ml LB broth; 400 μl of the overnight culture was inoculated into 20 ml prewarmed LB broth and cultivated at 28° with shaking (150 rpm) for 30 min. Tetracycline was added at the final concentration of 10 μg ml^{-1} to inhibit the growth of cells that had lost pUIC3. After growth for 2 hr, cycloserine was added at 800 μg ml^{-1} and growth was continued for another 4 hr (during this step the growing Tc^R cells are killed). The cells were then washed in sterile water, diluted, and inoculated onto LB plus X-Gal plates. SBW25 strains carrying polar mutations were generated by SOE–PCR and allelic replacement as described above, but an Ω-Sp cassette [retrieved from plasmid pHP45Ω (Fellay *et al.* 1987)] was inserted in place of the deleted gene.

Complementation of the *hutH* genes was performed by cloning the PCR-amplified coding region of $hutH_1$ or $hutH_2$ into pME6010 (Heeb *et al.* 2000) at the *Eco*RI site. The plasmid was introduced into PBR801 (Δ$hutH_1/H_2$) by conjugation with the help of pRK2013 (Tra$^+$).

Construction of *lacZ* transcriptional fusions and assay for β-galactosidase activity: *lacZ* reporter fusions were generated by cloning an ~800-bp fragment in front of the promoterless '*lacZ* carried on the integration vector of pUIC3 (Rainey 1999). The resulting plasmid was mobilized into *P. fluorescens* SBW25 or derived mutant strains by conjugation with the help of pRK2013. Integration into the genome by insertion–duplication was selected on LB plates supplemented with Nf and Tc.

Expression of '*lacZ* fusions was measured by β-galactosidase assay using 4-methylumbelliferyl-β-d-galactoside (4MUG) as the enzymatic substrate. The product (7-hydroxy-4-methylcoumarin, 4MU) was detected using a Hoefer DyNA Quant 200 fluorometer (Pharmacia Biotech) following the manufacturer's instructions. The reaction was monitored at 460 nm with an excitation wavelength of 365 nm. Cell density was determined by measuring the absorbance of the culture at 600 nm. The enzyme activity was expressed as "aM 4MU min^{-1} cell^{-1}" (1 aM = 10^{-18} mol).

Rapid amplification of cDNA 5′-ends: Transcriptional start sites of the *hut* operons were determined using the rapid amplification of cDNA 5′-ends (5′-RACE) system (Invitrogen, Carlsbad, CA). Total RNA was isolated by using the TRIzol RNA extraction reagent (Invitrogen) from *P. fluorescens* SBW25 cells grown in M9 salt medium supplemented with histidine. Primers PhutU5 (5′-TGAGGACCCAACGGCCTTTG-3) and PhutU1 (5′-TCTGGCCGTACATGGCTAG-3′) were used for the cDNA synthesis and the subsequent nested PCR amplification of the *hutU* transcript. To identify the *hutF* transcriptional start, primers PhutC1 (5′-GAGTGGAAGCACAGGCCCA-3′) and PhutF1 (5′-TGATCTGACGCGACAGTTC-3′) were used. The final 5′-RACE products were purified in agarose gel and extracted by using QIAGEN's QIAquick gel extraction kit before being cloned into pCR8/GW/TOPO (Invitrogen). Ten randomly chosen colonies were analyzed by DNA sequencing.

Assessment of bacterial fitness in laboratory media and *in planta*: Performance of mutant strains growing in laboratory media and *in planta* was examined by direct competition with the *lacZ*-marked wild-type strain of *P. fluorescens* SBW25 (SBW25-*lacZ*). Competition experiments were initiated with a 1:1 ratio of each strain [acclimated for 48 hr prior to initiating competition (see above)]. The initial frequency was determined by dilution plating onto LB plus X-Gal plates. For performance in laboratory medium, 5 μl of the bacterial suspension was inoculated into 5 ml of the tested medium in a 20-ml plastic

TABLE 1

Bacterial strains and plasmids

Strain or plasmid	Genotypes and relevant characteristics	Source or reference
P. fluorescens[a]		
SBW25	Wild-type strain isolated from sugar beet	Thompson *et al.* (1995)
SBW25-*lacZ*	SBW25 carrying '*lacZ* marker in a phage locus	X.-X. Zhang and P. B. Rainey
PBR800	*hutF*::Ω or *pflu0358*::Ω, insertion at nucleotide 423, Sp^R	This study
PBR801	Δ*hutH*$_1$/*H*$_2$	This study
PBR802	Δ*hutH*$_1$	This study
PBR803	Δ*hutH*$_2$	This study
PBR804	*hutC*::Ω, insertion at nucleotide 266, Sp^R	This study
PBR805	Δ*hutC*	This study
PBR806	Δ*hutD*	This study
PBR807	Δ*hutCD*	This study
PBR808	Δ*rpoN*	J. Jones and G. Preston
PBR809	Δ*cbrA*	This study
PBR810	Δ*cbrB*	This study
PBR811	DUP(*hutC-hutF*)::pUIC3, the *hutF-'lacZ* fusion strain of wild-type SBW25, Tc^R	This study
PBR812	DUP(*hutF-hutC*)::pUIC3, the *hutC-'lacZ* fusion strain of wild-type SBW25, Tc^R	This study
PBR813	DUP(*hutD-hutU*)::pUIC3, the *hutU-'lacZ* fusion strain of wild-type SBW25, Tc^R	This study
PBR814	DUP(*hutI-hutG*)::pUIC3, the *hutG-'lacZ* fusion strain of wild-type SBW25, Tc^R	This study
PBR815	*hutU*::Ω, insertion at nucleotide 633, Sp^R	This study
PBR816	*hutC*::Ω DUP(*hutF-hutC*)::pUIC3, *hutC-'lacZ*, Sp^R, Tc^R	This study
PBR817	*hutC*::Ω DUP(*hutD-hutU*)::pUIC3, *hutU-'lacZ*, Sp^R, Tc^R	This study
PBR818	*hutC*::Ω DUP(*hutI-hutG*)::pUIC3, *hutG-'lacZ*, Sp^R, Tc^R	This study
PBR819	*hutU*::Ω DUP(*hutI-hutG*)::pUIC3, *hutG-'lacZ*, Sp^R, Tc^R	This study
PBR820	Δ*hutH*$_1$/*H*$_2$ DUP(*hutC-hutF*)::pUIC3, *hutF-'lacZ*, Tc^R	This study
PBR821	Δ*hutH*$_1$/*H*$_2$ DUP(*hutF-hutC*)::pUIC3, *hutC-'lacZ*, Tc^R	This study
PBR822	Δ*hutH*$_1$/*H*$_2$ DUP(*hutD-hutU*)::pUIC3, *hutU-'lacZ*, Tc^R	This study
PBR823	Δ*hutC* DUP(*hutD-hutU*)::pUIC3, *hutU-'lacZ*, Tc^R	This study
PBR824	Δ*hutD* DUP(*hutD-hutU*)::pUIC3, *hutU-'lacZ*, Tc^R	This study
PBR825	Δ*hutCD* DUP(*hutD-hutU*)::pUIC3, *hutU-'lacZ*, Tc^R	This study
PBR832	*hutU*::Ω DUP(*hutD-hutU*)::pUIC3, *hutU-'lacZ*, Tc^R	This study
Plasmid		
pRK2013	Helper plasmid, Tra^+, Km^R	Ditta *et al.* (1980)
pCR2.1	Cloning vector, Ap^R, Km^R	Invitrogen
pCR8/GW/TOPO	Cloning vector, Sp^R	Invitrogen
pHP45Ω-Sp	Source of the Ω-Sp cassette, Sp^R	Fellay *et al.* (1987)
pME6010	Broad-host-range cloning vector, Tc^R	Heeb *et al.* (2000)
pME6010-*hutH*$_1$	*hutH*$_1$ construct for complementation, Tc^R	This study
pME6010-*hutH*$_2$	*hutH*$_2$ construct for complementation, Tc^R	This study
pUIC3	Integration vector with promoterless '*lacZ*, Mob^+, Tc^R	Rainey (1999)
pUIC3-2	pUIC3::(*hutD-hutF*), the *hutF-'lacZ* fusion plasmid, Tc^R	This study
pUIC3-3	pUIC3::(*hutF-hutD*), the *hutC-'lacZ* fusion plasmid, Tc^R	This study
pUIC3-8	pUIC3::(*hutD-hutU*), the *hutU-'lacZ* fusion plasmid, Tc^R	This study
pUIC3-12	pUIC3::(*hutI-hutG*), the *hutG-'lacZ* fusion plasmid, Tc^R	This study

[a] The *hut-'lacZ* fusion strain was constructed by cloning the DNA fragment into the delivery vector pUIC3 followed by integration into the *hut* locus by insertion–duplication. Thus the *hut* gene function was not affected.

tube. After growth for 24 hr, it was subcultured at a 1000× dilution. After two transfers (~20 generations), final ratios were determined by counting colonies on LB plus X-Gal plates. Relative fitness was expressed in terms of the selection rate constant (SRC) (Lenski 1991). A plant competitive colonization assay was performed as previously described (Zhang *et al.* 2006).

RESULTS

Genetic identification of *pflu0358* as *hutF*: Interrogation of the genome sequence of *P. fluorescens* SBW25 using the amino acid sequence of proteins known to be involved in histidine metabolism revealed a cluster of 12 genes (*hutC–hutG*) (Figure 1, Table 2). In addition to the core degradative enzymes (HutHUIG), the SBW25 *hut* operon harbors a second copy of the histidase gene (*hutH*) along with five open reading frames (ORFs) that are predicted to play a role in uptake: *plfu0363–0365* are likely to encode an ABC-type transport system and *pflu0362* and *pflu0368* encode predicted permeases [*pflu0368* is required for histidine utilization (Zhang *et al.* 2006)].

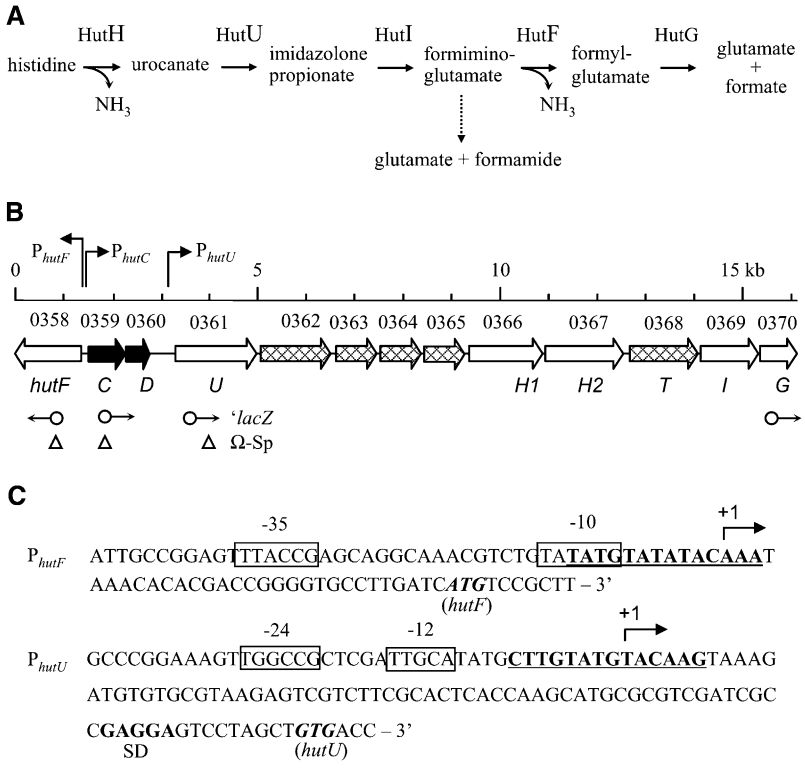

FIGURE 1.—The histidine degradation pathways (A) and structure of the *P. fluorescens* SBW25 *hut* locus (B) with details of the promoter regions of P_{hutF} and P_{hutU} (C). (A) The five-step histidine degradation pathway of Pseudomonas (HU *et al.* 1987) is shown by solid arrow with gene product involved in each reaction: HutH, histidine ammonia-lyase or histidase; HutU, urocanase; HutI, imidazolone propionate (IPA) amidohydrolase; HutF, formiminoglutamate (FIGLU) iminohydrolase; HutG, formylglutamate (FG) amidohydrolase. The divergent final step of the four-enzyme pathway (MAGASANIK 1978) is indicated by a dashed arrow and it is catalyzed by FIGLU formiminohydrolase. The gene encoding this enzyme has been referred to as *hutG*; however, it shares little in sequence identity with the *hutG* from Pseudomonas and thus it is not shown in the diagram to avoid confusion. (B) The metabolic *hut* genes and the *hut* regulators are shown by open and solid arrows, respectively. Crosshatched arrows represent putative transporters. ORF numbers are derived from the current SBW25 genome annotation (http://www.sanger.ac.uk/Projects/P_fluorescens). Insertion sites of the Ω-Sp cassette are indicated by open triangles. Location and orientation of the three histidine-induced promoters are indicated by bent arrows. Positions of the *lacZ* fusions are shown by open circles with attached arrow. (C) The putative HutC-binding sites are marked with underlined letters in boldface type. They were identified by their sequence similarities with the known HutC-binding site (CTTGTACATACAAG) from *P. putida* (HU *et al.* 1989). Transcriptional start site determined by 5′-RACE experiments is indicated by +1.

The ORFs immediately adjacent to the *hut* locus were examined to see whether any might be assigned a role in histidine uptake or metabolism. ORF *pflu0358* encodes a predicted protein of 454 amino acids (Figure 1). It contains a conserved amidohydrolase domain (Pfam01979, *E*-value $8e^{-5}$) and belongs to a group of metallo-dependent hydrolases with unknown function (cd01313, *E*-value $1e^{-141}$). The possibility that this gene might encode FIGLU iminohydrolase (HutF) was tested by construction of a mutant PBR800 (*pflu0358*::Ω). PBR800 grew normally on minimal-M9 medium with glucose and ammonia as carbon and nitrogen sources, but was incapable of growing when histidine (or urocanate) was the sole carbon and nitrogen source. Next, a *lacZ* transcriptional fusion was constructed to *pflu0358* and integrated into the genome of SBW25 in such a way as to ensure that *pflu0358* function was not affected. The resulting fusion strain (PBR811) was grown in minimal-M9 medium (with glucose and ammonia) supplemented with histidine or urocanate and β-galactosidase activity was measured. Results showed that *pflu0358* transcription was elevated 12-fold by the addition of either histidine or urocanate (mean aM 4MU min^{-1} $cell^{-1}$ ± standard error): 0.49 ± 0.18 in M9, 5.67 ± 0.83 in M9 plus histidine, and 8.57 ± 3.22 in M9 plus urocanate.

A search of sequenced Pseudomonas genomes shows that in each genome *hutC* lies adjacent to a homolog of *pflu0358*, but oriented in the opposite direction. Furthermore, a homolog of *pflu0358* exists in *S. coelicolor* A3(2), a bacterium, which like *P. putida* (Table 2), has a five-enzyme histidine degradation pathway (KENDRICK and WHEELIS 1982; CONSEVAGE *et al.* 1985). No homologs were identified in the genomes of bacteria known to possess the four-enzyme degradation pathway, for example, *S. typhimurium, K. pneumoniae* (MAGASANIK 1978), or *B. subtilis* (CHASIN and MAGASANIK 1968). Together these data are consistent with the genetic organization of *hutF* reported in *P. putida* ATCC 12633 (CONSEVAGE *et al.* 1985) and indicate that *pflu0358* is *hutF*. Further evidence was provided by the HutF amino acid sequence from *P. putida* ATCC 12633 (A. PHILLIPS, unpublished data) and *P. aeruginosa* PAO1 (MARTI-ARBONA *et al.* 2006), which show 84 and 76% sequence identity, respectively, to the deduced amino acid sequence of *hutF* from *P. fluorescens* SBW25.

Functional characterization of the *hutH*-like genes: The *hut* locus of SBW25 harbors two copies of *hutH*-like genes ($hutH_1$ and $hutH_2$), which encode proteins that show 36 and 84% sequence identity with the biochemically characterized HutH from *P. putida* (Table 2). Interestingly two copies of HutH-like genes are also found within the *hut* locus of all the genome-sequenced Pseudomonas strains (*P. syringae* DC3000, 1448A, and B728a; *P. aeruginosa* PAO1; and *P. fluorescens* Pf0-1 and Pf-5) with the exception of *P. putida* KT2440. To investigate functionality, both copies of *hutH* were deleted from the SBW25 genome. The mutant strain PBR801 (Δ$hutH_1$/H_2) was unable to grow on histidine, but could grow on

TABLE 2

The *P. fluorescens* SBW25 *hut* genes, predicted functions and similarity with homologs from *P. putida* ATCC 12633, KT2440, and *P. aeruginosa* PAO1

Gene[a]	Product	Amino acid sequence identity with homolog in other Pseudomonas[b]		
		P. putida		*P. aeruginosa*:
		ATCC 12633 (%)[c]	KT2440 (%)[d]	PAO1 (%)[e]
0359 (*hutC*)	Repressor	HutC (83)	PP5035 (83)	PA5105 (84)
0360 (*hutD*)	Hypothetical protein	ORF2 (63)	PP5034 (63)	PA5104 (54)
0361 (*hutU*)	Urocanase	HutU (91)	PP5033 (92)	HutU (91)
0362	Putative permease			PA5099 (79)
0363	ABC-type transporter			PA5096 (77)
0364	ABC-type transporter			PA5095 (90)
0365	ABC-type transporter			PA5094 (87)
0366 (*hutH$_1$*)	Putative histidase	HutH (36)	PP5032 (38)	PA5093 (76)
0367 (*hutH$_2$*)	Putative histidase	HutH (84)	PP5032 (97)	HutH (81)
0368 (*hutT*)	Histidine transporter	HutT (84)	PP5031 (86)	HutT (78)
0369 (*hutI*)	Imidazolone propionate (IPA) hydrolase	HutI (70)	PP5030 (80)	HutI (78)
0370 (*hutG*)	Formyl-glutamata (FG) amidohydrolase	HutG (75)	PP5029 (73)	HutG (72)
0358 (*hutF*[f])	Formimino-glutamata (FIGLU) iminohydrolase	Not available	PP5036 (82)	PA5106 (76)

[a] The *hut* genes are predicted on the basis of sequence from the unannotated *P. fluorescens* SBW25 genome sequence (http://www.sanger.ac.uk).

[b] Identity of the deduced amino acid sequences is shown in parentheses.

[c] The *hut* locus in *P. putida* ATCC 12633 has been characterized by Phillips and co-workers. Sequences were retrieved from the NCBI database by the following accession numbers: GI409364 (*hutU*), GI2642338 (*hutTIG*), GI151273 (*hutH*), and GI151275 (*hutCD*).

[d] The *hut* locus of *P. putida* KT2440 has not been experimentally characterized.

[e] Gene names (*hut*) are shown for those that have been functionally characterized by mutational analysis (Rietsch *et al.* 2004).

[f] The *hutF* (*pflu0358*) was identified in this study by mutational analysis.

urocanate, which confirms the histidase (histidine ammonia-lyase) function of *hutH$_1$* and/or *hutH$_2$*. Next, single-deletion mutants PBR802 (Δ*hutH$_1$*) and PBR803 (Δ*hutH$_2$*) were produced: deletion of *hutH$_1$* had no effect; however, deletion of *hutH$_2$* resulted in a strain unable to grow on histidine. Additionally, complementation of PBR801 was achieved by introduction of a cloned copy of *hutH$_2$*, but not *hutH$_1$*. Analysis of the deduced amino acid sequences of *hutH$_1$* and *hutH$_2$* showed that HutH$_1$ lacks the conserved Ala–Ser–Gly active site residues (Schwede *et al.* 1999) consistent with the genetic data that indicate that *hutH$_2$* (but not *hutH$_1$*) encodes a functional histidase.

In *P. putida* ATCC 12633, urocanate, rather than histidine, is the physiological inducer of *hut* genes (Allison and Phillips 1990). If this also holds for SBW25, then histidine should be incapable of activating *hut* transcription in a mutant of SBW25 lacking histidase function. To this end, induction of *hutF* transcription was examined in the double *hutH$_1$/H$_2$* mutant (PBR801) using the chromosomally integrated *hutF*-'*lacZ* fusion (fusion strain PBR820). Histidine was no longer capable of inducing *hutF* expression, whereas urocanate induction was not significantly affected (mean aM 4MU min^{-1} cell^{-1} ± standard error): 0.29 ± 0.01 in M9, 0.51 ± 0.17 in M9 plus histidine, and 3.59 ± 0.27 in M9 plus urocanate. Therefore, both histidine and urocanate induce *hut* expression, but urocanate is the direct inducer.

Transcriptional organization of the *hut* genes: *hutF* must be transcribed as a single gene unit from a promoter between *hutF* and *hutC* (P_{hutF}). However, the transcriptional organization of the remaining 12 *hut* genes was uncertain (Figure 1B). To test whether the 12 *hut* genes from *hutC* to *hutG* are transcribed as a single mRNA, a *lacZ* fusion was made to the first (*hutC*) and the last (*hutG*) of the 12 genes (to give fusion strains PBR812 and PBR814, respectively), and their response to urocanate determined. In the wild-type background, both *hutC* and *hutG* were induced by the presence of urocanate (Table 3, PBR812 and PBR814). This result confirms the existence of a urocanate-inducible promoter in the front of *hutC* (P_{hutC}).

To test for the existence of additional promoter(s) downstream of *hutC*, a *hutC* polar mutant, PBR804, was generated (*hutC*::Ω). In both *hutC*::Ω and wild-type backgrounds, *hutG* expression was elevated in the presence of urocanate compared to the wild-type *hutG*-'*lacZ* fusion grown in M9 (Table 3, fusion strains PBR818 and

TABLE 3

Urocanate-induced *hut* gene expression in strains that carry polar mutations of the *hut* loci

Fusion strain	Genetic background	β-Galactosidase (aM 4MU min^{-1} $cell^{-1}$)[a] M9	β-Galactosidase (aM 4MU min^{-1} $cell^{-1}$)[a] M9 + urocanate[b]
PBR812	*hutC*-'*lacZ*, wild type	0.88 ± 0.13	2.90 ± 0.43 (S)
PBR816	*hutC*-'*lacZ*, *hutC*::Ω	5.37 ± 0.53	4.80 ± 0.42 (NS)
PBR813	*hutU*-'*lacZ*, wild type	1.69 ± 0.04	27.65 ± 1.33 (S)
PBR817	*hutU*-'*lacZ*, *hutC*::Ω	81.38 ± 3.15	43.66 ± 3.83 (S)
PBR832	*hutU*-'*lacZ*, *hutU*::Ω	8.12 ± 0.48	55.59 ± 2.85 (S)
PBR814	*hutG*-'*lacZ*, wild type	0.96 ± 0.11	7.42 ± 1.83 (S)
PBR818	*hutG*-'*lacZ*, *hutC*::Ω	34.48 ± 3.13	33.22 ± 9.49 (NS)
PBR819	*hutG*-'*lacZ*, *hutU*::Ω	1.45 ± 0.07	1.30 ± 0.35 (NS)

[a] β-Galactosidase activities were measured for cells growing in minimal-M9 medium (M9) and M9 supplemented with urocanate (M9 + urocanate).

[b] Data are means and standard errors of three independent cultures from eight independent experiments. Each experiment was designed to test the hypothesis that the fusion is urocanate inducible. Results of paired *t*-tests are provided: S and NS indicate significant and nonsignificant induction ($P < 0.05$), respectively.

PBR814). The fact that *hutG* transcription was not abolished by the *hutC* polar mutation indicates the presence of a second transcriptional unit. To localize the predicted additional *hut* promoter(s), the intergenic regions located downstream of *hutC* were examined. The TGA stop codon of *hutC* overlaps the ATG start codon of the adjacent gene (*pflu0360*), suggesting cotranscription. Between *pflu0360* and *hutU* lies a 405-nucleotide region that could potentially define a promoter. To test this possibility, a *lacZ* fusion to *hutU* was constructed in *hutC*::Ω and expression was measured by β-galactosidase assay. In both the *hutC*::Ω and wild-type backgrounds, *hutU* transcription was elevated in the presence of urocanate (Table 3, fusion strains PBR813 and PBR817). The *hutC* polar mutation did not abolish *hutU* expression in the presence of urocanate, indicating the existence of a second transcriptional unit initiated from a promoter immediately upstream of *hutU* (P_{hutU}).

To determine the existence of additional promoter(s) between *hutU* and *hutG*, a *hutU* polar mutation was generated (PBR815). Unlike PBR804 (*hutC*::Ω), PBR815 (*hutU*::Ω) was unable to grow on either histidine or urocanate (*hutU* encodes urocanase; in addition the omega cassette has polar effects). As shown in Table 3, *hutG*-'*lacZ* expression was not induced by urocanate in the *hutU*::Ω genetic background (fusion strain PBR819) while the positive control (*hutU*-'*lacZ*) remained urocanate inducible (fusion strain PBR832). This shows that there is no functional urocanate-inducible promoter between *hutU* and *hutG*.

Taken together, the genetic data show that the *hut* locus is organized into three transcriptional units: *hutF*, *hutCD*, and *hutU–G* (Figure 1B).

Data on the effect of *hutC* inactivation on the transcription of *hutC*, *hutU* and *hutG* (Table 3), and *hutF* (not shown) are consistent with HutC from SBW25 also functioning as a repressor of *hut* gene transcription (Hu *et al.* 1989). To determine whether urocanate is the direct inducer of *hutCD* and *hutU–G* (in addition to *hutF*), histidine and urocanate-induced expression of *hutCD* and *hutU–G* was determined in the Δ*hutH*$_1$/*H*$_2$ genetic background using '*lacZ* fusions to *hutC* and *hutU* (fusion strains PBR821 and PBR822, respectively). Expression was stimulated by urocanate, but not by histidine (mean aM 4MU min^{-1} $cell^{-1}$ ± standard error): *hutC* expression was 0.41 ± 0.2 in M9, 0.38 ± 0.04 in M9 plus histidine, and 1.76 ± 0.15 in M9 plus urocanate; *hutU* expression was 2.08 ± 0.22 in M9, 2.23 ± 0.67 in M9 plus histidine, and 16.7 ± 0.51 in M9 plus urocanate. Urocanate is therefore the physiological inducer of all three *hut* operons.

Determination of the *hut* transcriptional start sites: To identify the promoter sequences of P_{hutF} and P_{hutU} (the two putative promoters that control expression of *hut* structural genes) 5′-RACE was used to identify the 5′-end sequence of each transcript from cells grown on minimal-M9 medium with histidine as the sole source of carbon and nitrogen (Figure 1C). Immediately upstream of the *hutF* transcriptional start site resides a sequence of nucleotides (TTACCG N_{16} TATATG) that is similar to the σ^{70}-promoter consensus (TTGACA $N_{16–18}$ TATAAT). In addition, a putative HutC binding site (Hu *et al.* 1989) overlaps the −10 region of P_{hutF} (Figure 1C).

When the nucleotide sequence upstream of the *hutU* transcriptional start was examined, a motif (TGGCCG N5 TTGCA) was identified that shows strong similarity to the σ^{54}-promoter −24/−12 consensus (TGGCAC N5 TTGCW). However, the start site is the 14th nucleotide downstream from the conserved C residue of the −12 element (Figure 1C), whereas transcription from such promoters is typically initiated at the 12th nucleotide downstream from the conserved C residue of the −12 element. This atypical spacing has been reported for other σ^{54}-dependent promoters (Barrios *et al.* 1999). Also present in the vicinity of the *hutU* transcriptional start is a putative HutC binding site (Figure 1C) (Hu *et al.* 1989).

Transcriptional activation of *hut* requires σ^{54} and CbrB: The observation that *hut* genes in the *hutU–G* operon are controlled by a σ^{54}-dependent promoter suggested the involvement of both σ^{54} and a σ^{54} activator (Wosten 1998). In Pseudomonas, σ^{54} is encoded by *rpoN*; thus a *rpoN* deletion mutant (PBR808, a gift from Jake Jones and Gail Preston) was examined for its ability to utilize histidine and urocanate. PBR808 grew normally in minimal-M9-salt medium supplemented with glucose and ammonia, but was unable to grow in minimal-M9-salt medium with histidine (or urocanate) as the sole carbon and nitrogen source. This demonstrates the requirement of σ^{54} for utilization of histidine and urocanate. It also shows that derepression of HutC (by plating cells in the presence of either histidine or urocanate) is a necessary, but not sufficient, requirement for activation of *hut* transcription.

Next we sought the identity of the σ^{54} activator. A possible candidate was indicated by Nishijyo *et al.* (2001) who showed that in *P. aeruginosa* PAO1 a two-component regulatory system, CbrAB, was associated with *hut* regulation. Significantly, the response regulator CbrB contains a σ^{54}-type regulator output domain in addition to a signal-receiver domain. *In silico* analysis of the *P. fluorescens* SBW25 genome revealed a two-gene locus (*pflu5236* and *pflu5237*) that shows significant similarity to the CbrAB genes of *P. aeruginosa* PAO1. The genetic organization of this locus in SBW25 (hereafter referred to as SBW25 *cbrAB*) mirrors that found in PAO1: it is composed of two genes, orientated in the same direction, and separated by 22 nucleotides.

To test the role of CbrAB in *hut* regulation a *cbrA* and a *cbrB* mutant were constructed (PBR809 and PBR810, respectively) and their ability to utilize histidine and urocanate as sole carbon and nitrogen sources was determined. Both PBR809 and PBR810 grew normally in M9 salt medium supplemented with glutamate (the end product of the histidine degradation pathway), but were unable to grow in M9 salt medium supplemented with histidine or urocanate. This result, in conjunction with the *in silico* analysis data, implicates CbrB as a σ^{54}-enhancer binding protein required for transcriptional activation of the *hutU–G* operon.

Functional investigation of the hypothetical protein Pflu0360 (HutD): The TGA stop codon of *hutC* overlaps the ATG start codon of a downstream ORF (*pflu0360*). This organization suggests that transcription of *pflu0360* is coordinated with *hutC* and that *pflu0360* may play a role in *hut* regulation, possibly in conjunction with HutC. We designate this open reading frame *hutD* (Figure 1) on the basis of the functional characterization described below.

Interrogation of the NCBI protein database with the deduced amino acid sequence of *hutD* provided no clues as to its function: it belongs to a group of uncharacterized proteins that are highly conserved in bacteria (Pfam0596; DUF886, COG 3758). A recently determined crystal structure of HutD (PA5104) from *P. aeruginosa* PAO1 (PDB.1yll) reveals no insight into function. In Pseudomonas, *hutD* is always located downstream of *hutC* in an overlapped manner, whereas in other gram-negative bacteria, *e.g.*, *Burkholderia cenocepacia*, *Mesorhizobium loti*, *Yersinia pestis*, and *Serratia marcescens*, the *hutD* homolog is located in the *hut* locus but not adjacent to *hutC*.

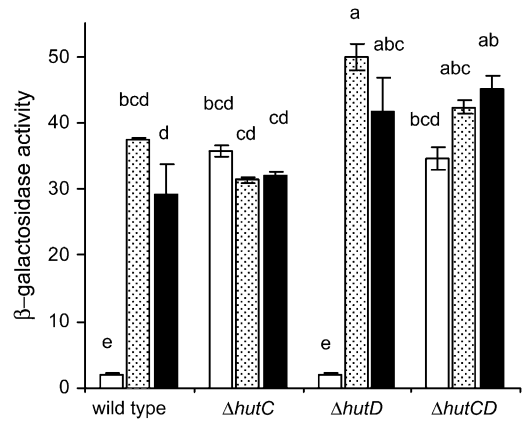

Figure 2.—Expression of *hutU* in wild-type SBW25 and the *hutCD* mutants. β-Galactosidase activities (aM 4MU min^{-1} $cell^{-1}$) were measured in *hutU-'lacZ* fusion strains PBR813 (wild type), PBR823 (Δ*hutC*), PBR824 (Δ*hutD*), and PBR825 (Δ*hutCD*). Bacteria were grown in M9 salts plus glucose and ammonia (open bars), M9 salts plus urocanate (stippled bars), and M9 salts plus histidine (solid bars). Values are means and standard errors of three independent cultures. Bars that are not connected by the same letter (shown above each) are significantly different ($P < 0.05$) by Tukey's HSD.

To investigate the role of *hutD* and possible interactions with *hutC*, three in-frame deletion mutants were generated: PBR805 (Δ*hutC*), PBR806 (Δ*hutD*), and PBR807 (Δ*hutCD*). The three mutant strains were subjected to phenotypic assays to determine: first, effects on transcription of the *hutU–G* operon; second, ability to grow on histidine (and urocanate) as a sole source of carbon and nitrogen; and third, the contribution of each mutation to fitness in laboratory media.

Expression of the *hutU–G* operon was measured using a chromosomally integrated *hutU-'lacZ* fusion in the genetic background of wild-type SBW25 (as a control; PBR813) and the *hutC* (fusion strain PBR823), *hutD* (fusion strain PBR824), and *hutCD* mutants (PBR825). β-Galactosidase was assayed for cells growing in minimal-M9 medium supplemented with glucose and ammonia, histidine, or urocanate. Results are shown in Figure 2. Consistent with previous findings (see above), the *hutU–G* operon was constitutively expressed in a Δ*hutC* background (fusion strain PBR823). The operon was also constitutively expressed in Δ*hutCD* (fusion strain PBR825), whereas in Δ*hutD* (fusion strain PBR806) and in the wild-type background (PBR813), the *hut* operon remained histidine and urocanate inducible.

In M9 salt medium supplemented with glucose and ammonia, the basal level of *hutU–G* expression was negligible in the wild-type background and was not affected by the *hutD* deletion. However, a statistically significant increase in *hutU–G* transcription (attributable to *hutD*) was detected when *hutU–G* expression was compared in the wild-type and the *hutD* mutant strains (grown on M9 salt medium plus histidine or urocanate, fusion strains PBR813 and PBR824). A similar effect, also attributable to *hutD*, was evident when *hutU–G* transcription was compared in *hutC vs. hutCD* mutant strains (see Figure 2, PBR823 and PBR825). From these data we conclude that HutC is the sole *hut* repressor; HutD appears to limit the upper level of transcriptional induction.

Next we examined the growth properties of the mutants in minimal-M9 medium supplemented with glucose and ammonia, histidine, or urocanate (Figure 3 and Table 4). The three mutant strains showed similar growth characteristics to wild-type SBW25 when grown on minimal-M9 medium supplemented with glucose and ammonia, although PBR805 (Δ*hutC*) and PBR807 (Δ*hutCD*) mutants showed slower growth, a likely consequence of constitutive expression of enzymes in an environment where they are not required (Savageau 1989, 1998).

When grown on either histidine or urocanate as the sole carbon and nitrogen source, growth of the *hutD* mutant strain PBR806 was significantly impaired: the lag time was extended by ~4 hr and the maximum-growth rate (μmax) was ~20% lower than the wild type (Table 4). The opposite effect was observed in the *hutC* mutant (PBR805): this genotype showed more rapid growth than the wild type, presumably because there is no delay in activating *hut* gene expression (Savageau 1989). PBR807 (Δ*hutCD*) displayed an intermediate phenotype (Figure 3).

To further analyze the growth phenotypes, particularly the slow growth of PBR806 (Δ*hutD*) on histidine or urocanate, the competitive ability of these mutants relative to the wild-type ancestor was determined in three shaken-broth cultures containing minimal-M9 medium and supplemented with (1) glucose and ammonia, (2) histidine, or (3) urocanate. The mutant was mixed 1:1 with a *lacZ*-marked "wild-type" strain (SBW25-*lacZ*) and the bacterial mixture was inoculated into test media. After growing in competition for ~20 generations the ratio of mutant to the wild-type competitor was determined by plating onto LB plates supplemented with X-Gal. As a control for any possible effects due to the *lacZ* marker, ancestral wild-type SBW25 was competed in parallel against the SBW25-*lacZ*. Results are shown in Figure 4A and closely parallel the trends revealed from the analysis of the growth of individual strains (Figure 3, Table 4). However, when histidine or urocanate was the sole carbon and nitrogen source, the fitness of PBR806 (Δ*hutD*) and PBR807 (Δ*hutCD*) was drastically impaired relative to wild type. The fitness of

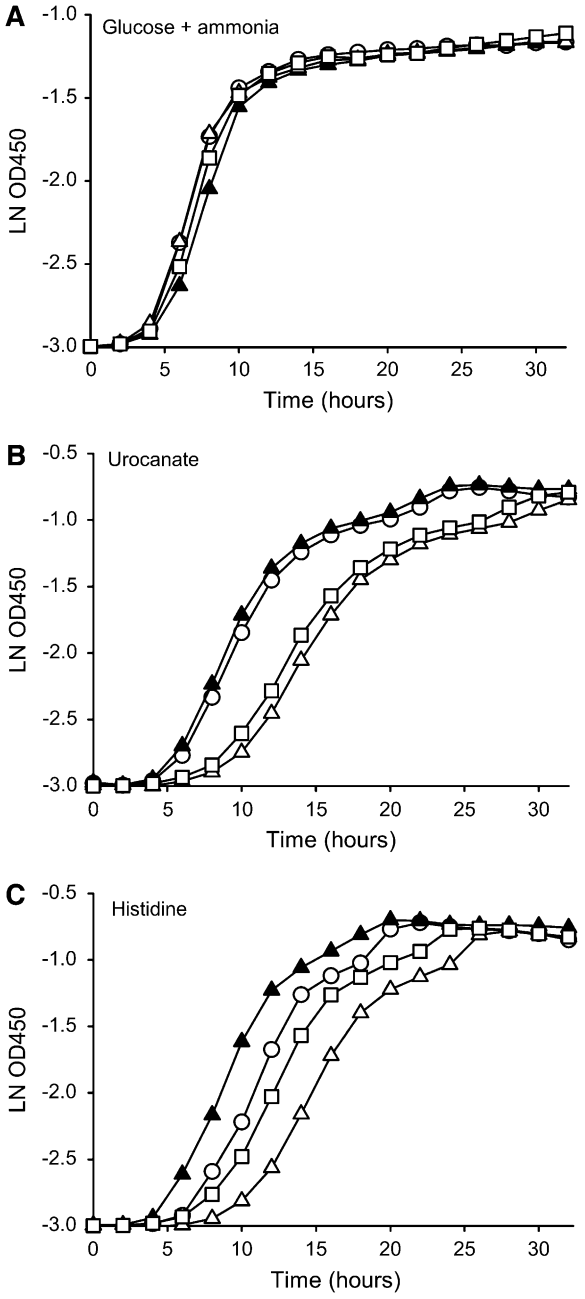

Figure 3.—Growth dynamics of the *hutCD* deletion mutants. Growth was measured for wild-type SBW25 (open circles) and mutants PBR805 (Δ*hutC*, solid triangles), PBR806 (Δ*hutD*, open triangles), and PBR807 (Δ*hutCD*, open squares) in M9 (M9 salts plus glucose and ammonia, A), M9 salts plus urocanate (B) and M9 salts plus histidine (C). Results are means of eight independent cultures. Data were collected at 5-min intervals, but two hourly time points are shown for clarity. Standard errors are within the symbols and thus not visible.

PBR805 (Δ*hutC*) was not impaired; indeed, PBR805 (Δ*hutC*) was more fit than wild type on minimal-M9-salt medium supplemented with histidine. The fact that deletion of *hutD* produced a phenotype equivalent to wild type in the histidine-free environment (and distinct from Δ*hutC* and Δ*hutCD*), but equivalent to Δ*hutCD* in

TABLE 4

Maximum growth rate (μmax) of the *hutCD* mutants when growing in laboratory media

Strain (genotype)	Minimal-M9-salt medium supplemented with[a]		
	Glucose and ammonia	Urocanate	Histidine
SBW25 (wild type)	0.425 ± 0.006 (a)	0.350 ± 0.006 (b)	0.342 ± 0.007 (b)
PBR805 (Δ*hutC*)	0.412 ± 0.008 (a)	0.357 ± 0.007 (b)	0.350 ± 0.011 (b)
PBR806 (Δ*hutD*)	0.431 ± 0.006 (a)	0.259 ± 0.003 (c)	0.274 ± 0.004 (c)
PBR807 (Δ*hutCD*)	0.430 ± 0.012 (a)	0.259 ± 0.007 (c)	0.294 ± 0.006 (c)

[a] Two-way ANOVA revealed a highly significant difference among means ($F_{2,83} = 310.48$, $P < 0.0001$). Growth rates (μmax) identified by different letters are significantly different ($P < 0.05$) by Tukey's HSD.

the histidine-containing environment indicates that *hutC* and *hutD* are not functionally interdependent.

The observed fitness effects are consistent with the known repressor function of HutC and further suggest that HutD may function to govern the upper level of *hut* activation. To examine these effects more closely, the fitness of each of the three mutants was determined on M9 salt medium supplemented with 5 mM glutamate (the end product of histidine degradation and itself a source of carbon and nitrogen), but containing different concentrations of histidine (from 0.5 mM to 15 mM). If HutD functions as a governor that limits the upper level of *hut* expression, then the fitness cost associated with deletion of *hutD* ought to increase with increasing histidine concentration. Conversely, the fitness cost associated with derepression of *hut* (brought about by deletion of *hutC*) should decrease with increasing histidine concentration (Savageau 1989, 1998). Assuming that *hutC* and *hutD* function independently of one another and given opposing fitness effects associated with deletion of each gene, then fitness of a Δ*hutCD* mutant should be low at both low and high concentrations of histidine. The results shown in Figure 4B are fully consistent with these predictions.

Ecological significance of *hut* regulatory genes in plant environment: The competitive ability of each mutant [PBR805 (Δ*hutC*), PBR806 (Δ*hutD*), and PBR807 (Δ*hutCD*)] relative to the *lacZ*-marked "wild-type" strain (SBW25-*lacZ*) was determined during the course of colonization of sugar beet seedlings. After a 2-week period of competitive colonization, bacteria were recovered from the shoot and rhizosphere and the fitness of each mutant was determined relative to wild type.

The fitness of PBR805 (Δ*hutC*) and PBR807 (Δ*hutCD*) was significantly impaired in the plant environment, whereas PBR806 (Δ*hutD*) was not affected (Figure 5). Drawing upon the data shown in Figure 4 (and its interpretation) the data of fitness in the plant environment indicate that histidine is scarce in this environment—a finding consistent with previous measures that showed histidine to be present in the rhizosphere at ~3 μM (Zhang *et al.* 2006). The fitness of PBR815 (*hutU*::Ω) was indistinguishable from wild type (Figure 5), which indicates that neither histidine nor urocanate are important sources of carbon or nitrogen in the plant environment.

DISCUSSION

Amino acids are a significant source of carbon and nitrogen in many terrestrial and marine environments (Jaeger *et al.* 1999; Phillips *et al.* 2004). Not surprisingly then, bacteria possess specific pathways dedicated to the uptake and degradation of specific amino acids. The histidine uptake and utilization pathway of SBW25 is typical of these pathways.

Our genetic analysis has confirmed previously known aspects of the function of the *hut* pathway, but has also extended understanding of gene composition, organization, and regulation. Of value has been the bringing together of various aspects of *hut* genetics within a single study to lay the foundations for further work. Indeed, our original goal in focusing attention on *hut* had been to study the function of the five predicted uptake components (Zhang *et al.* 2006), but this proved impossible without the genetics of *hut* first achieving a contemporary position.

In terms of *hut* metabolic genes the work reported here makes two new contributions. The first concerns the identity and genomic location of *hutF*, a gene long presumed to exist within organisms that degrade histidine by the five-step enzymatic pathway (Consevage *et al.* 1985), but that had escaped identification because the link between DNA sequence, amino acid sequence, and enzyme activity had not been made (Janiyani and Ray 2002; Rietsch *et al.* 2004).

Recently, Marti-Arbona *et al.* (2006) characterized three proteins from *P. aeruginosa* (PA5106, PA5091, and PA3175) with predicted roles in the breakdown of formiminoglutamate. Chemical analysis showed that PA5106 and PA5091 are HutF and HutG, which catalyze the last two steps of the five-enzyme pathway: the steps formiminoglutamate to formylglutamate (and ammonia), and formylglutamate to glutamate (and formate), respectively (Figure 1A). Interestingly, the gene product of PA3175 is able to catalyze the hydrolysis of formiminoglutamate to glutamate and formamide, the end reaction of the four-step *hut* pathway (Marti-Arbona

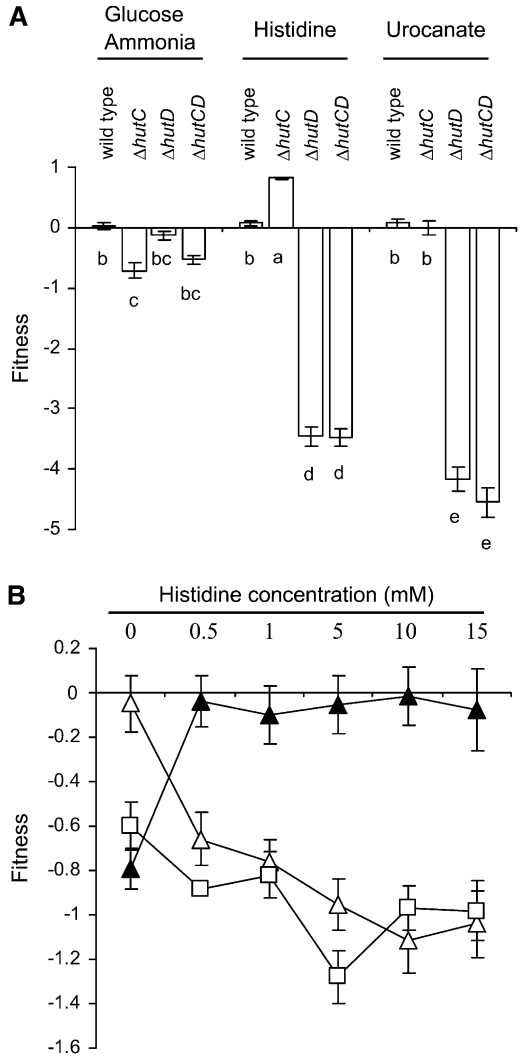

Figure 4.—Fitness of the *hutCD* deletion mutants relative to *lacZ*-marked *P. fluorescens* SBW25 in laboratory media. (A) Fitness of SBW25 (wild type), PBR805 (Δ*hutC*), PBR806 (Δ*hutD*), and PBR807 (Δ*hutCD*) relative to SBW25-*lacZ* grown in M9 medium, or M9 salts medium with histidine or urocanate as sole carbon and nitrogen sources. Data are means and standard errors of 10 independent cultures. A fitness of zero indicates that the fitness of the mutant is identical to wild type (a negative value indicates a reduction in fitness relative to wild type). (B) Fitness of PBR805 (Δ*hutC*, solid triangles), PBR806 (Δ*hutD*, open triangles), and PBR807 (Δ*hutCD*, open squares) relative to SBW25-*lacZ* grown on M9 salts medium supplemented with 5 mM glutamate and varying concentrations of histidine. Data are means and standard errors of six independent cultures.

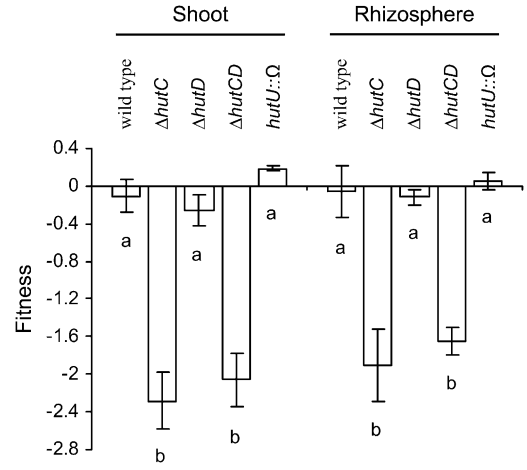

Figure 5.—Fitness of the *hutCD* deletion mutants relative to *lacZ*-marked *P. fluorescens* SBW25 on sugar beet seedlings. Data are shown in an order of strains: ancestral SBW25 (wild type), PBR805 (Δ*hutC*), PBR806 (Δ*hutD*), and PBR807 (Δ*hutCD*) and PBR815 (*hutU*::Ω). Fitness was determined after 2 weeks of competitive colonization. Results are means and standard errors of data collected from the shoot and rhizosphere of eight plants, which were inoculated by independent cultures. A fitness of zero indicates that the fitness of the mutant is identical to wild type (a negative value indicates a reduction in fitness relative to wild type).

et al. 2006). This finding, based solely on *in vitro* assays of enzyme activity, led to the suggestion that *P. aeruginosa* PAO1 may be capable of degrading histidine by both the four- and the five-step pathways (Marti-Arbona *et al.* 2006). Our mutant analyses suggest that this is unlikely. The *hut* operons of PAO1 and SBW25 are highly similar (Table 1); furthermore, SBW25 contains a homolog of PA3175 (Pflu4510: 42% amino acid similarity). If Pflu4510 is expressed, and functional, then PBR800 (*hutF*::Ω) ought not be compromised in its ability to grow on histidine. The fact that PBR800 (*hutF*::Ω) is unable to grow on histidine indicates that SBW25 degrades histidine, at least under the conditions of the assay, solely by the five-step pathway. By extension, the same is likely to be true of *P. aeruginosa*.

The second contribution stems from analysis of the two copies of *hutH*, both of which are predicted to encode histidine ammonia lyase or histidase. Our data show that $hutH_1$ is incapable of complementing a $hutH_1/H_2$ double mutant even when expressed from a constitutive promoter. This indicates that the protein is not a functional histidine ammonia lyase. The fact that the *hut* locus from six of the seven genome-sequenced Pseudomonas species/strains also carries an additional copy of *hutH* is puzzling; moreover, the high degree of conservation of the *hutH1* homologs [all lack the exact same three amino acid residues (Ser–Gly–Asp) that span the active site and share between 76 and 86% amino acid identity (X.-X. Zhang and P. B. Rainey, unpublished data)] adds to the mystery. A number of evolutionary scenarios can be envisaged, perhaps the most plausible being an ancient duplication or gene-acquisition event (which sits comfortably with the fact that HutH1 and HutH2 share only 37% sequence identity); although given the significant similarity among $HutH_1$ homologs it would be necessary to invoke a single inactivation event in a single ancestral strain. An additional possibility is that $hutH_1$ has an as yet undetermined functional role in the metabolism of histidine, for example, as a protein that binds (but does not metabolize)

histidine. Such a role may be related to the need to control the upper limit of *hut* activity (see below).

In terms of regulation, *hut* is more complex and interesting than anticipated (Phillips and Mulfinger 1981). In all respects our work supports the previous claims that HutC is a repressor of *hut* (importantly, we experimentally demonstrated the involvement of HutC in the expression of each of the three operons) and that urocanate is the immediate inducer of expression (of all three operons); however, identification of the promoter sequences of P_{hutF} and P_{hutU} provided evidence of additional control by σ^{70} and σ^{54}, respectively. Involvement of σ^{54} led to a search for a likely σ^{54}-activator protein, the most obvious candidate being CbrB, a response regulator with a σ^{54}-type regulator output domain (Nishijyo *et al.* 2001). While DNA binding studies are required to demonstrate the direct connection between CbrB and *hut* transcription, the evidence gathered (experimental and *in silico*) implicates CbrB as the σ^{54} enhancer binding protein for transcriptional activation of the *hutU–G* operon. The fact that *hutU–G* is regulated by both σ^{54} and HutC (plus urocanate) provides a rare example of a σ^{54}-dependent operon that is subject to both general and specific regulation (Reitzer and Schneider 2001).

Analysis of *hutCD* demonstrates a further complexity to *hut* regulation: inactivation of *hutD* shows that it is required for efficient utilization of histidine (and urocanate) as a sole carbon and nitrogen source. The fact that *hutD* is translationally coupled to *hutC*, combined with the fact that HutD shows no homology to known histidine metabolic genes, suggests that HutD plays a regulatory role.

The combined *hutC*, *hutD*, and *hutCD* analyses show that across the range of growth and fitness assays the Δ*hutCD* genotype expressed a phenotype that was not consistently that of either the Δ*hutC* or the Δ*hutD* genotypes. In fact the *hutC* mutation was dominant to the *hutD* mutation in environments without histidine, but the reverse was observed in environments replete with histidine. This suggests that HutD acts independently of HutC. Independent action is consistent with an early report that showed that the protein encoded by the *hutD* homolog (*orf2*) from *P. putida* ATCC12633 was not required for binding of HutC to the operator site in front of *hutU* (Allison and Phillips 1990). Such a finding is also consistent with the fact that HutD lacks a helix-turn-helix domain and thus it is unlikely to act by binding to DNA.

In addition to evidence of independent action, the transcriptional, growth, and fitness assays suggest a more specific role for HutD, possibly as a governor of *hut* transcription. Such a role would be analogous to, although mechanistically distinct from, the "governor" site at the *glnAp2* promoter of glutamine synthase (GS) in *E. coli*, which serves to limit the maximum activity of the GS promoter (Atkinson *et al.* 2002). The precise function of HutD remains to be determined but it is possible that HutD may limit *hut* activation by controlling the intracellular concentration of the *hut* inducer, urocanate, perhaps by binding this compound and thus preventing the intracellular concentration of inducer from exceeding a critical threshold level. Computational analysis based on the deposited crystal structure of HutD (PA5104) from *P. aeruginosa* PAO1 (PDB.1yll) indicates that urocanate, but not histidine, docks with the active site of HutD (V. Arcus, unpublished data). The biological need for a governor may relate to the potentially harmful effects that could result from an excess of intracellular ammonia, a likely consequence of too high a rate of histidine metabolism.

The complexity of *hut* regulation suggests that the metabolism of histidine is of central metabolic importance to Pseudomonas. Drawing upon Savageau's demand theory of regulatory control (Savageau 1974, 1989, 1998), negative regulation indicates that the *hut* operon is infrequently used and that histidine is scarce in the natural environment of this bacterium. Indeed, the scarcity of histidine in the plant rhizosphere has previously been shown (Zhang *et al.* 2006) and is also indicated here. Puzzling then is the positive control by CbrB because this suggests that histidine is frequently encountered in the environment. We cautiously suggest that the apparent paradox can be explained by envisaging a more general role for CbrAB, for example, as a sensor of a range of different amino acids and as a positive activator for a number of specific amino acid degradation pathways, a proposal for which there is some published evidence (Nishijyo *et al.* 2001; Knight *et al.* 2006) and for which we have additional data (X.-X. Zhang, D. G. Brown and P. B. Rainey, unpublished data).

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