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The origin and ecological significance of multiple branches for histidine utilization in *Pseudomonas aeruginosa* PAO1

Monica L. Gerth,[1]* Matteo P. Ferla[2] and Paul B. Rainey[1,3]

[1]*New Zealand Institute for Advanced Study and*
[2]*Institute of Natural Sciences, Massey University, Auckland, New Zealand.*
[3]*Max Planck Institute for Evolutionary Biology, Plön, Germany.*

Summary

Pseudomonas **proliferate in a wide spectrum of harsh and variable environments. In many of these environments, amino acids, such as histidine, are a valuable source of carbon, nitrogen and energy. Here, we demonstrate that the histidine uptake and utilization (*hut*) pathway of *Pseudomonas aeruginosa* PAO1 contains two branches from the intermediate formiminoglutamate to the product glutamate. Genetic analysis revealed that the four-step route is dispensable as long as the five-step route is present (and vice versa). Mutants with deletions of either the four-step (HutE) or five-step (HutFG) branches were competed against each other and the wild-type strain to test the hypothesis of ecological redundancy; that is, that the presence of two pathways confers no benefit beyond that delivered by the individual pathways. Fitness assays performed under several environmental conditions led us to reject this hypothesis; the four-step pathway can provide an advantage when histidine is the sole carbon source. An IclR-type regulator (HutR) was identified that regulates the four-step pathway. Comparison of sequenced genomes revealed that *P. aeruginosa* strains and *P. fluorescens* Pf-5 have branched *hut* pathways. Phylogenetic analyses suggests that the gene encoding formiminoglutamase (*hutE*) was acquired by horizontal gene transfer from a *Ralstonia*-like ancestor. Potential barriers to interspecies transfer of the *hutRE* module were explored by transferring it from *P. aeruginosa* PAO1 to *P. fluorescens* SBW25. Transfer of the operon conferred the ability to utilize histidine via the four-step pathway in a single step, but the fitness cost of acquiring this new operon was found to be environment dependent.**

Received 4 October, 2011; accepted 2 December, 2011. *For correspondence. E-mail m.l.gerth@massey.ac.nz; Tel. (+64) 9414 0800 ext. 41518; Fax (+64) 9441 8136.

Introduction

Redundancy is a frequently encountered property of biological systems (Edelman and Gally, 2001). From a theoretical perspective two main types of redundancy are recognized. The first, referred to as full redundancy, is mediated by gene duplicates that perform the same catalytic reaction (Gu *et al*., 2003; Dean *et al*., 2008). The second, referred to as degeneracy or distributed robustness, encompasses those situations where different pathways fulfil similar or partially overlapping functions (Marx *et al*., 2004; Denef *et al*., 2006; Bazurto and Downs, 2011). It is this second type of redundancy that is thought to have greatest relevance to living systems, giving contributions to both robustness and evolvability (Edelman and Gally, 2001; Whitacre and Bender, 2010). While the advantages of redundancy are readily understandable, testing the hypothesis that two components are functionally equivalent – especially where those two components are non-homologous – poses numerous challenges. In the context of genetic or metabolic systems, two components may be interchangeable in one environment, but confer distinct advantages or disadvantages in another.

Pseudomonas aeruginosa PAO1 has a large genome of 6.3 million base pairs and can thrive in diverse ecological niches including soil, water and plant or animal tissues (Stover *et al*., 2000). Both large genome sizes and versatility of lifestyle are thought to correlate with high levels of metabolic network redundancy (Mahadevan and Lovley, 2008; Soyer and Pfeiffer, 2010; Mendonça *et al*., 2011).

Histidine, like all amino acids, is an important source of carbon, nitrogen and energy for *Pseudomonas*. In *P. aeruginosa*, *P. fluorescens* and *P. putida* – *Pseudomonas* species where histidine degradation has been the subject of intensive investigation – degradation proceeds via a 'five-step' pathway (Fig. 1) (Tabor and Mehler, 1954; Lessie and Neidhardt, 1967; Hu *et al*., 1987; Zhang and Rainey, 2007a). However, in many other bacteria, for

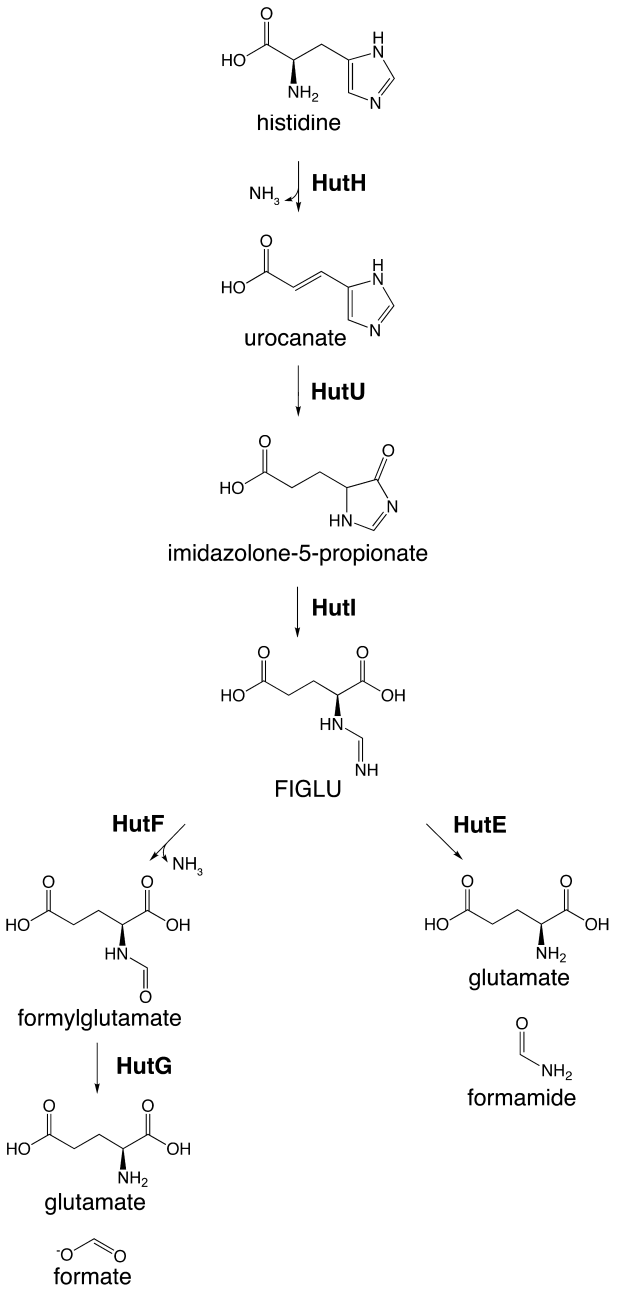

Fig. 1. The four- and five-step pathways for histidine catabolism. The first three steps are common to both pathways and are catalysed by the following enzymes: histidine ammonia lyase (HutH, EC 4.3.1.3); urocanase (HutU, EC 4.2.1.49); and imidazolone propionate amidohydrolase (HutI, EC 3.5.2.7). In the five-step pathway, formiminoglutamate deiminase (HutF, EC 3.5.3.13) and formylglutamate amidohydrolase (HutG, EC 3.5.1.68) complete the degradation of *N*-formimino-L-glutamate (FIGLU) to yield L-glutamate and formate. In the four-step pathway, formiminoglutaminase (HutE, EC 3.5.3.8) directly converts FIGLU to L-glutamate and formamide in a single step. It should be noted that traditionally formiminoglutamase has also been referred to as HutG, or occasionally as HutG to distinguish it from the HutG of *Pseudomonas* (Itoh *et al*., 2007). Since formiminoglutamase is structurally and mechanistically unrelated to *N*-formyl-glutamate deformylase, for the sake of clarity we refer to the gene encoding formiminoglutamase as *hutE* and the 4-step pathway as the *hutHUIE* system.

example, in *Salmonella*, *Klebsiella* and *Bacillus*, degradation proceeds via a 'four-step' pathway (Kimhi and Magasanik, 1970; Smith *et al*., 1971; Rodriguez and West, 1984). The four- and five-step pathways share the first three enzymes [histidine ammonia lyase (HutH); urocanase (HutU); and imidazolone propionate amidohydrolase (HutI)], but diverge at the intermediate formiminoglutamate (FIGLU). In *Pseudomonas*, formiminoglutamate deiminase (HutF) and formylglutamate amidohydrolase (HutG) complete the conversion of FIGLU to glutamate (Fig. 1). In contrast, organisms with the four-step pathway possess a single enzyme [formiminoglutamase (HutE)] that achieves this conversion. In addition to glutamate, both pathways yield a one-carbon unit (formate in the five-step, and formamide in the four-step). The five-step pathway also yields an additional molecule of ammonia compared with the four-step pathway (Itoh *et al*., 2007).

The ecological and evolutionary significance of the two pathways is unclear. While their existence may be nothing more than an accident of history (two ways to achieve the same ends), the fact that the five-step pathway delivers an additional molecule of ammonia has led some to suggest that the more efficient nitrogen utilization by the five-step HutFG route might have favoured the evolution of this alternative pathway (Hu and Phillips, 1988; Itoh *et al*., 2007).

To date, the existence of both four- and five-step pathways for the degradation of histidine in a single bacterium has not been reported: the question of redundancy has therefore not been raised. However, recently a gene from *P. aeruginosa* PAO1 was shown to encode a protein that has formiminoglutamase activity *in vitro* (Marti-Arbona *et al*., 2006). While the subject of debate (Marti-Arbona *et al*., 2006; Itoh *et al*., 2007; Zhang and Rainey, 2007a; Johnson *et al*., 2008) this finding suggests that *P. aeruginosa* possesses both the four- and five-step pathways. If true, then this hints at metabolic redundancy, but a plausible competing hypothesis would be that the two pathways confer advantages under different ecological conditions (Harrison *et al*., 2007; Papp *et al*., 2009). For example, *Escherichia coli* has two pathways for glutamate synthesis: one is important when energy is abundant or the external concentration of ammonia is low; the other is used when the cell is limited for energy (and carbon), but ammonium is present in excess (Helling, 1994).

From both evolutionary and functional perspectives, distinguishing between competing hypotheses is important; it is also important from a therapeutic perspective. Catabolic pathways are potential targets for inhibiting

pathogenesis. It has been shown that the loss of amino acid utilization pathways impacts the *in vivo* proliferation of some pathogenic bacteria (Velayudhan *et al*., 2004), including *P. aeruginosa* PAO1 (Boulette *et al*., 2009). Genes involved in histidine utilization have been linked to biofilm development (Musken *et al*., 2010; Patell *et al*., 2010), swarming motility (Yeung *et al*., 2009) and adaptation to the host environment (Mashburn *et al*., 2005; Alvarez-Ortega and Harwood, 2007; Fung *et al*., 2010). Catabolism of histidine also influences expression of the type III secretion system (Rietsch *et al*., 2004).

Here, we report genetic studies demonstrating that the putative formiminoglutamase of *P. aeruginosa* PAO1 is functional *in vivo*. *P. aeruginosa* PAO1 thus has two routes for the degradation of FIGLU. Construction of mutant strains with either the four-step or the five-step route allowed a test of the hypothesis that the two pathways are ecologically equivalent. Under nitrogen-limited conditions both pathways delivered equivalent benefit; however, under carbon-limited conditions the four-step pathway resulted in superior ecological performance. To understand the origins of the four-step pathway we performed a comparative phylogenetic analysis and provide evidence that the five-step pathway is the ancestral state, whereas the four-step pathway is the result of horizontal gene transfer (HGT) events. Finally, we show that transfer of the four-step pathway's *hutRE* operon from *P. aeruginosa* to *P. fluorescens* is sufficient to allow degradation of histidine via a four-step pathway.

Results and discussion

Two routes from FIGLU to glutamate are functional in P. aeruginosa *PAO1*

The existence in *P. aeruginosa* PAO1 of the full suite of enzymes necessary for degradation of histidine via the five-step pathway (Stover *et al*., 2000; Winsor *et al*., 2009), combined with recent evidence that this bacterium also contains a gene encoding a protein that has formiminoglutamase activity (Marti-Arbona *et al*., 2006), suggests two pathways for the conversion of FIGLU to glutamate. To test this hypothesis three mutants were constructed: one lacking both the five- and putative four-step pathways [Δ*hutE*Δ*hutF*Δ*hutG* (PBR1022)]; one lacking the five-step pathway [Δ*hutF*Δ*hutG* (PBR1021)]; and one lacking the putative four-step pathway [Δ*hutE* (PBR1020)]. Growth of these mutants was measured in Minimal Medium P (MMP) with glucose and ammonia and in MMP with histidine as the sole carbon and nitrogen source.

All three deletion strains were indistinguishable from wild-type PAO1 when grown in MMP with glucose and ammonia (Fig. S1). However, growth of PBR1022 (which lacks both branches) was completely abolished in MMP with histidine as the sole carbon and nitrogen source (Fig. 2). PBR1021 and PBR1020 (lacking the five- and four-step branches respectively) both had similar growth rates and final cell densities as the wild-type strain (Fig. 2) when grown in MMP with histidine as sole carbon and nitrogen source. These data demonstrate that *P. aeruginosa* PAO1 has both four- and five-step routes for the degradation of histidine and that both are active *in vivo*. In addition, the failure of PBR1022 to grow indicates the existence of two (and only two) routes for metabolizing FIGLU to glutamate.

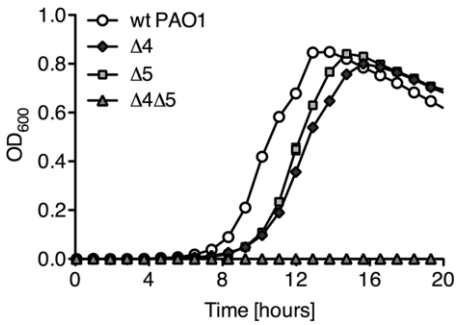

Fig. 2. The growth of *hut* pathway deletion mutants demonstrates that both the four- and five-step routes for histidine utilization are active *in vivo*. Growth of wild-type *P. aeruginosa* PAO1 (PBR1019, circles), a mutant with the *hutE* branch of the putative four-step pathway deleted (PBR1020, diamonds), a mutant with the *hutFG* branch of the five-step pathway deleted (PBR1021, squares) and a double mutant with both the *hutE* and *hutFG* branches deleted (PBR1022, triangles) was measured in minimal medium with 20 mM histidine (as the sole carbon and nitrogen source). Data are the means and standard errors calculated from three independent experiments; if not visible, the error bars are contained within the symbol.

Ecological significance of four- versus five-step pathways

The existence of two interchangeable, but non-homologous, pathways for histidine degradation is evidence of functional redundancy. However, a key issue is whether the pathways are ecologically equivalent. Seemingly dispensable genes may be important under specific ecological conditions (though predicting the appropriate conditions can be difficult). Our previous knowledge of fitness differences attributable to carbon and nitrogen utilization led us to consider the possibility that one difference between the two paths is the amount of ammonia (and therefore nitrogen) generated. The HutFG route generates two molecules of ammonia from histidine, while the HutE route generates just one molecule of ammonia (Fig. 1) (Itoh *et al*., 2007). This led us to predict that under nitrogen-limited conditions, the five-step pathway confers an advantage over the four-step pathway.

Table 1. Relative fitness of *hut* pathway mutants in different environments.

Competitor 1	Competitor 2	Environment	$W \pm$ SE[a]
Four-step pathway[b]	Wild-type[c]	20 mM glucose, 20 mM histidine	0.98 ± 0.02
Five-step pathway[d]	Wild-type	See above	1.00 ± 0.01
Four-step pathway	Five-step pathway[e]	See above	0.99 ± 0.02
Four-step pathway	Wild-type	20 mM glucose, 1 mM histidine	1.01 ± 0.01
Five-step pathway	Wild-type	See above	1.00 ± 0.02
Four-step pathway	Five-step pathway	See above	1.01 ± 0.01
Four-step pathway	Wild-type	20 mM histidine, 20 mM ammonia	0.98 ± 0.02
Five-step pathway	Wild-type	See above	0.86 ± 0.01*
Four-step pathway	Five-step pathway	See above	1.15 ± 0.02*
Four-step pathway	Wild-type	20 mM histidine, 1 mM ammonia	1.02 ± 0.01
Five-step pathway	Wild-type	See above	0.88 ± 0.02*
Four-step pathway	Five-step pathway	See above	1.14 ± 0.02*
Four-step pathway	Wild-type	20 mM histidine	0.91 ± 0.02*
Five-step pathway	Wild-type	See above	0.82 ± 0.02*
Four-step pathway	Five-step pathway	See above	1.32 ± 0.02*

a. Fitness (W) values depict the fitness of Competitor 1 relative to Competitor 2 and are expressed as mean ± SE ($n = 8$). $W = 1.0$ indicates no difference in fitness between the two competitors. Values greater than 1 indicate a higher fitness of the first competitor relative to the second.
b. PBR1021.
c. PBR1023.
d. PBR1020.
e. PBR1024.
*Significance level of $P < 0.05$ for a two-tailed test with the null hypothesis that $W = 1$, calculated using the t distribution and 7 d.f.

To test this hypothesis, the fitness of four-step and five-step only variants was compared by competing the two strains under a range of environmental conditions. In addition, each single pathway variant was competed against the wild-type ancestor in the same environment. A number of minimal medium environments were tested in which the availability of histidine relative to other carbon and nitrogen sources was varied: histidine as the sole carbon and nitrogen source; histidine as the sole nitrogen source (at both high concentrations and limiting concentrations); and histidine as the sole carbon source (at both high and limiting concentrations).

Results of competitive fitness assays when histidine was the sole nitrogen source are shown in Table 1. In both competitive assays, glucose was available as a carbon source (at 20 mM), while histidine was provided at 20 mM or 1 mM. Contrary to expectation, the strain harbouring the five-step pathway showed no fitness advantage relative to the strain harbouring just the four-step pathway – despite the extra ammonia molecule generated by the five-step pathway. At the lower concentration of histidine (1 mM) cell growth was limited by the lack of available nitrogen (the strains reached a maximum density after 24 h that was approximately 4.5-fold lower than the strains propagated in medium with 20 mM histidine). Even under these nitrogen limited conditions, there was no observable difference in the fitness of any of the competitors.

However, fitness differences between the pathway variants were evident in competitions where histidine was provided as the sole carbon source. As shown in Table 1, when histidine was the carbon source the strain using the four-step pathway was significantly more fit than the five-step pathway strain (i.e. $W > 1$; $P < 0.05$). No statistically significant difference was observed when the four-step variant was competed against wild-type PAO1. In contrast, the five-step pathway strain was much less fit than wild-type PAO1 (which has both the four- and five-step branches present). In all assays with histidine as the sole carbon source, the overall results of the competitive fitness assays were the same, regardless of the histidine concentration provided in the medium (20 mM or 1 mM).

The results of competitive fitness assays when histidine was the sole carbon and nitrogen source (Table 1), show that the four-step pathway variant has a fitness advantage compared with the five-step variant ($W = 1.32$). Each single pathway variant was less fit when competed against the wild-type ancestor (with both pathways present). The mutant with only the five-step pathway was at the greatest fitness disadvantage ($W = 0.82$) when histidine (20 mM) was the sole carbon and nitrogen source.

The fitness of the pathway variants was also tested in a defined synthetic cystic fibrosis sputum medium (SCFM), which mimics the nutritionally rich growth environment of CF sputum (Palmer *et al.*, 2007). The concentration of histidine in this medium is 0.5 mM. In this environment, there was no difference in the fitness of any of the strains (data not shown).

Overall, we found no advantage associated with the five-step pathway under any of the conditions tested. Instead, our results show that the four-step pathway provides a competitive advantage when histidine is the sole carbon source.

While we can only speculate as to the underlying cause of the advantage of the four-step pathway under conditions of energy limitation, it has been shown that in general, metabolic pathways have evolved to have the least number of steps (Meléndez-Hevia *et al*., 1994; Beasley and Planes, 2007). It may be that the shorter, four-step pathway, is more efficient (i.e. achieving the same product with fewer enzymes), or simply that the product (and energy source – glutamate) is reached more quickly than the longer five-step pathway, thus providing an advantage when in direct competition. The lack of benefit provided by the five-step pathway under conditions of nitrogen limitation goes against predictions (Itoh *et al*., 2007). The results of our fitness assays suggest that the nitrogen yield of each pathway is actually equivalent. In organisms that degrade histidine solely via the four-step pathway, formamide is excreted and not further metabolized (Neidhardt and Magasanik, 1957; Magasanik *et al*., 1965; Kaminskas *et al*., 1970). However, the ability to hydrolyse formamide to formate and ammonia has been observed in some *P. aeruginosa* strains (Kelly and Clarke, 1962; Clarke, 1969). Furthermore, we note that there is a putative aliphatic amidase (locus PA3366) in the genome of *P. aeruginosa* PAO1 (Stover *et al*., 2000; Winsor *et al*., 2009). While outside the scope of our present study, this would be an intriguing enzyme to study further.

Expression of the hutE *branch is dependent on a novel regulator*

The *hutE* gene is not colocalized with the canonical *hut* locus. There are no other known *hut* related genes in its genomic vicinity. Analysis of the *P. aeruginosa* PAO1 genome (Stover *et al*., 2000) shows that a gene (PA3174) encoding a putative transcriptional regulator is upstream from *hutE*, and separated by an intergenic region of 15 bp. On the basis of *in silico* analyses, the gene is predicted to form a two-gene operon with *hutE* [Database of prOkaryotic OpeRons (DOOR), operon ID 13762] (Dam *et al*., 2007)

Amino acid sequence analysis of the PA3174 gene product (hereafter referred to as HutR) suggests that it is a member of the IclR family of transcriptional regulators. The gene encodes a predicted protein of 242 amino acids with two domains: an N-terminal helix–turn–helix DNA-binding domain (Pfam 09339, E-value $1e^{-14}$) and an IclR-type C-terminal effector-binding domain (Pfam 01614, E-value $1e^{-10}$). The IclR family is diverse, with more than 500 members of sequence-related transcriptional regulators identified in bacterial and archaeal genomes (Krell *et al*., 2006). Characterized IclR-type regulators have been shown to function as repressors, or activators, or regulatory proteins with dual functions (Molina-Henares *et al*., 2006). The characterized members of this family are involved in the regulation of a variety of metabolic pathways, although no members of the IclR protein family are known to be involved in the regulation of histidine catabolism.

Online neural network promoter prediction (Reese, 2001) was used to identify a putative promoter with a transcription start site in the intergenic region that precedes *hutR*. This putative promoter region was cloned into the mini-Tn*7*T-*lacZ* transcriptional reporter vector (Choi and Schweizer, 2006) and integrated into the PAO1 genome at the neutral *attB* site to generate PBR1025. β-Galactosidase activity was then monitored in the presence and absence of potential inducers. All available intermediates of the *hut* pathway were tested, including histidine, urocanate, FIGLU and formylglutamate. It was not possible to test imidazolone propionate as it is both unstable (Bochner and Savageau, 1979) and unavailable commercially. Histidine, urocanate and FIGLU all induced expression from the P_{hutR} promoter, by approximately 30-fold (Fig. 3A). Formylglutamate had no significant effect (as compared with minimal medium, $P > 0.05$ by Tukey's HSD). Based on these results, we propose that FIGLU is the specific inducer of *hutRE* expression, and that histidine and urocanate are indirect inducers (requiring conversion to FIGLU before induction).

The transcriptional fusion was also used to investigate the mode of regulation. To distinguish between positive and negative modes of action, P_{hutR}-*lacZ* expression was compared in the wild-type and Δ*hutR* genetic backgrounds (PBR1025 and PBR1027 respectively). If HutR acts as a repressor, the promoter activity of P_{hutR}-*lacZ* should be constitutive in the Δ*hutR* mutant. Conversely, if HutR is an activator, promoter activity should be reduced in a Δ*hutR* genetic background. β-Galactosidase activity was measured in cells grown in MMP (with glucose and ammonia) in the presence and absence of FIGLU (20 mM). The results (Fig. 3B) show that the promoter activity was significantly reduced in the Δ*hutR* deletion mutant. This indicates that HutR is a transcriptional activator. This was further tested by measuring the ability of a Δ*hutR* strain to utilize histidine via the four-step (*hutE*) route. Two strains with a deletion in this gene were constructed: *hutR* was deleted from the wild-type PAO1 strain and from the *hutFG* deletion strain to generate PBR1026 and PBR1028 respectively. This allowed us to test whether *hutR* is necessary for the utilization of histidine as a carbon source via the four-step (*hutE*) branch. While both the Δ*hutFG* strain and the Δ*hutR* strains could grow on histidine (Fig. S2), a combined mutant (Δ*hutFG* Δ*hutR*)

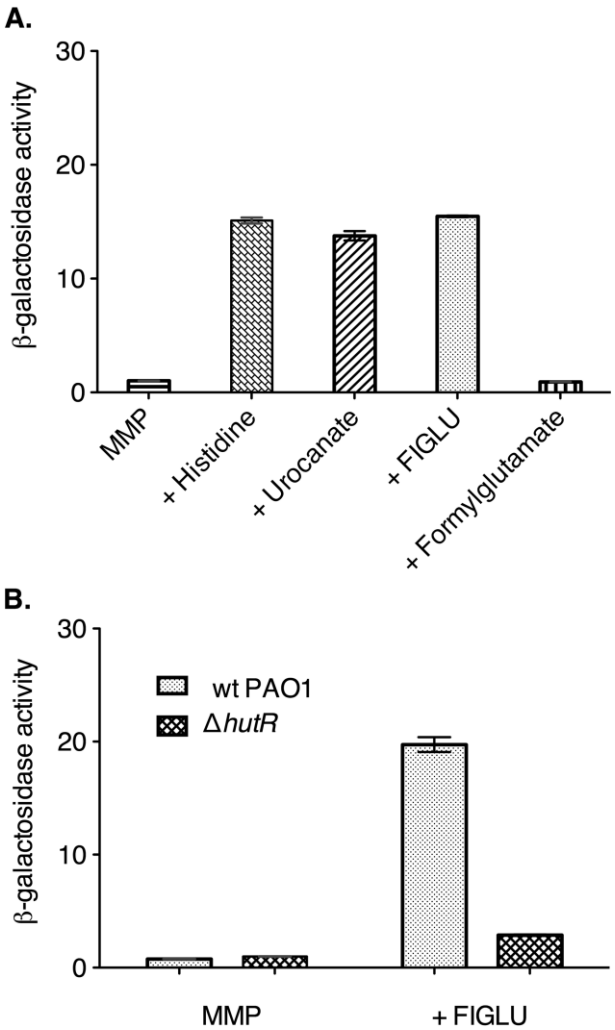

Fig. 3. Regulation of the HutE branch.
A. Effect of potential inducers on the *hutR* promoter. The *hutR* promoter region was fused to the *lacZ* gene and integrated into the chromosome of wild-type *P. aeruginosa* PAO1 to generate PBR1025 (PAO1::mini-Tn*7*T-P_{hutR}-*lacZ*). Cultures of PBR1025 were grown to mid-log phase in Minimal Medium P (MMP) with supplements as indicated at 20 mM.
B. *hutR* encodes a positive regulator. β-Galactosidase assays of wild-type *P. aeruginosa* PAO1 and Δ*hutR* strains in the presence of the inducer FIGLU.

no longer displayed any growth (even though the *hutE* gene was still present). These results are consistent with our model, in which *hutR* activates *hutE* expression. Both the mode of regulation (positive) and the inducer (FIGLU) suggest that expression of the *hutE* branch is only induced when the branch point intermediate (and *hutE* substrate) FIGLU is present.

Origin of the four-step pathway in Pseudomonas

With the exception of *P. stutzeri* A1501 and *P. mendocina* ymp, genes necessary for degradation of histidine via the five-step pathway are present in all genome-sequenced *Pseudomonas* species (there are currently complete sequences for eight species with multiple isolates for many species). The five-step pathway is thus likely to be ancestral to the *Pseudomonadaceae*. To explore this in greater detail a phylogenetic analysis was performed for each protein of the five-step pathway (both enzymatic and regulatory). In each instance, the *Pseudomonas* proteins show approximately the same relationship to their closest homologues (Figs S3–S9), which are from members of the *Enterobacteriales* (e.g. *Serratia*, *Yersinia* or *Klebsiella*) (Williams *et al*., 2010).

In contrast, *hutE* (and the adjacent linked gene, *hutR*) is found exclusively in *P. aeruginosa* (all four sequenced strains: PAO1, PA7, PA14 and LESB58), in *P. fluorescens* Pf-5 and in *P. mendocina* ymp. HutE from *P. mendocina* shares only 69% and 54% identity with the orthologous protein from *P. aeruginosa* PAO1 and *P. fluorescens* Pf-5 respectively. This sporadic distribution suggests that *hutE* (and *hutR*) are likely to have been acquired by HGT. Figure 4 shows the phylogenetic relationship among the 17 *Pseudomonas* strains – based on concatenation of 16S rDNA, *recA* and *gyrB* – for which genome sequences are available. Overlaid on this phylogeny is the distribution of *hutE*. If *hutE* is ancestral to this group of *Pseudomonas* species, then in order to explain the observed distribution of *hutE*, it would be necessary to invoke one HGT event (into the ancestor), followed by six independent gene-loss events. A more parsimonious explanation would invoke three independent HGT events: one into the ancestor of *P. aeruginosa*, one into *P. fluorescens* Pf-5 and one into *P. mendocina* ymp.

To further explore the likelihood of HGT, phylogenies of *hutE* and the linked gene *hutR* were constructed. *Pseudomonas hutE* genes do not cluster with dominant members of the *Enterobacteriales*, but instead are most similar to genes from the betaproteobacteria (‘*Aromatoleum*’ and *Ralstonia*) (Fig. S10). A similar analysis for the linked gene, *hutR*, also reveals little similarity to homologues from the *Enterobacteriales*: *hutR* shows overall greatest similarity to homologues found in the betaproteobacteria (Fig. S11). Together, these data support the hypothesis that the origin of *hutE* and *hutR* in *Pseudomonas* was HGT. In fact, the dissimilarity between *hutE* from *P. aeruginosa* and *P. fluorescens*, compared with *P. mendocina*, suggests the possibility of three independent HGT events; two mostly likely from a *Ralstonia*-like ancestor and the other, from a *Vibrio*-like ancestor.

Fitness effects of pathway acquisition

Together, HutE and HutR confer on *P. aeruginosa* PAO1 the ability to utilize histidine via the four-step pathway. Given the likelihood that the origin of HutE and HutR in

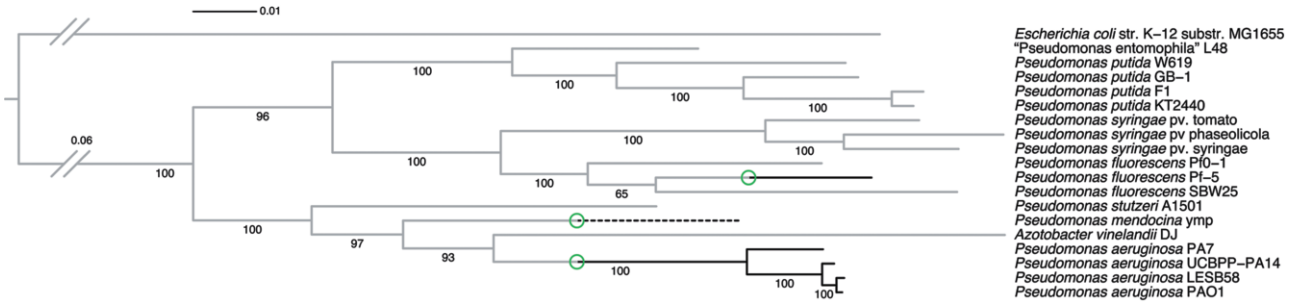

Fig. 4. Presence (black lines) or absence (grey lines) of *hutE* in the *Pseudomonas* 16S, *recA* and *gyrB* tree. In the phylogram, *Azotobacter vinelandii* is nested inside the *Pseudomonas* as expected (Rediers *et al*., 2004; Young and Park, 2007). Inferred HGT events are shown as green circles. All the sequenced *Pseudomonas* species, except *P. stutzeri*, possess histidine utilization genes. *P. aeruginosa* and only strain Pf-5 of *P. fluorescens* possess *hutE* and *hutR*, whereas *P. mendocina* ymp has a distantly related *hutE* and no *hutR* (dashed line). Numbers below branches indicate bootstrap support based on 1000 replicates.

Pseudomonas is attributable to HGT, the sporadic distribution of these two genes among *Pseudomonas* is of interest. While comparative genomics approaches have shown that HGT is the predominant form of genome expansion and metabolic innovation in prokaryotes (Ochman *et al*., 2000; Pal *et al*., 2005), there is a limited understanding of the immediate consequences of the incorporation of new functions through HGT.

To explore the consequences of inter-species HGT and potential barriers to acquisition, we transferred the *hutRE* operon that has been acquired by *P. aeruginosa* PAO1 to a *Pseudomonas* strain that lacks *hutRE*, that is, *P. fluorescens* SBW25. Both the phylogenetic work presented in this study and previous genetic analysis (Zhang and Rainey, 2007a) confirm that SBW25 does not have the *hutRE* branch, and that it degrades histidine solely by the five-step *hutHUIFG* pathway.

First, to test for functionality of *hutRE*, these two genes were introduced (using the mini Tn*7* system (Choi and Schweizer, 2006) into *P. fluorescens* SBW25 carrying a defect in the native five-step pathway [due to inactivation of *hutF* gene (PBR800) (Zhang and Rainey, 2007a)] and growth was determined on MMP with histidine as the sole carbon and nitrogen source. PBR800 carrying a defect in *hutF* failed to grow on histidine; however, growth was restored by introduction of the *hutRE* operon (PBR1029) (Fig. S12A). This demonstrated that *hutRE* was functional when transferred to *P. fluorescens* SBW25.

Next, the *hutRE* operon of *P. aeruginosa* PAO1 was transferred to wild-type *P. fluorescens* SBW25 to generate SBW25::*hutRE* (PBR1030). When competed against wild-type SBW25, in an environment with no histidine present, there was a significant fitness cost to carrying the foreign *hutRE* pathway ($W = 0.92 \pm 0.01$). In contrast, the fitness cost of the transferred *hutRE* module is neutral in environments where histidine is present (regardless of whether it was the carbon and/or nitrogen source) (Fig. S12B). Taken together, these results indicate that barriers to inter-species transfer for this module are small, and they are likely to be ameliorated readily by the presence of histidine in the environment.

Such a finding is consistent with previous studies, which show that upon initial acquisition, most horizontally transferred genes are likely to be deleterious – or at best neutral in effect (Berg and Kurland, 2002). Many aspects (expression, activity, regulation, etc.) of the transferred region may be suboptimal, especially with increasing phylogenetic distance between the donor and recipient organisms. Furthermore, new metabolic or catabolic pathways must be incorporated into the existing regulatory and metabolic network of the recipient (Lind *et al*., 2010).

In this instance the challenge is not to explain the presence of the operon in the environment in which it is needed (because there is no cost), but, rather, the maintenance of the operon in environments where it is not required. Perhaps the sporadic distribution of the four-step pathway reflects significant and perpetual differences in the kinds of environments that *P. aeruginosa* inhabits, compared with the environments in which other *Pseudomonas* species are found.

Experimental procedures

Bacterial strains, media and chemicals

Strains and plasmids used are shown in Table 2. Primer sequences are available upon request. *Escherichia coli* and *P. aeruginosa* PAO1 were grown at 37°C while *P. fluorescens* SBW25 was grown at 28°C. *Escherichia coli*, *P. aeruginosa* and *P. fluorescens* were routinely propagated in Luria–Bertani (LB) broth. *Pseudomonas* were also grown in minimal medium P (MMP), which contained 30 mM Na_2HPO_4, 14 mM KH_2PO4, 4 μM $FeSO_4$, 1 mM $MgSO_4$, 20 mM glucose and 20 mM NH_4Cl (Kwon and Lu, 2006). When used as a sole carbon and/or nitrogen source, histidine replaced glucose and/or ammonia in the medium at 20 mM. Antibiotics were used at the following concentrations for *E. coli* and/or *P. fluo-*

Table 2. Bacterial strains and plasmids used in this work.

Strain or plasmid	Genotype and/or characteristics[a]	Source or reference
P. aeruginosa strains		
PBR1019	Wild-type PAO1	I.L. Lamont
PBR1020	Δ*hutE*, deletion of PA3175	This work
PBR1021	Δ*hutF*Δ*hutG*, deletion of PA5106, PA5091	This work
PBR1022	Δ*hutE*Δ*hutF*Δ*hutG*, deletion of PA3175, PA5106, PA5091	This work
PBR1023	PAO1::mini-Tn*7*T- *lacZ*, wild-type strain marked with *lacZ*	This work
PBR1024	Δ*hutE* PAO1::mini-Tn*7*T- *lacZ*, Δ*hutE* strain marked with *lacZ*	This work
PBR1025	PAO1::mini-Tn*7*T-P_{hutR}-*lacZ*	This work
PBR1026	Δ*hutR*, deletion of PA3174	This work
PBR1027	Δ*hutR* PAO1::mini-Tn*7*T-P_{hutR}-*lacZ*	This work
PBR1028	Δ*hutR*Δ*hutF*Δ*hutG*, deletion of PA3174, PA5106, PA5091	This work
P. fluorescens strains		
SBW25	Wild-type strain isolated from sugar beet	Thompson *et al*. (1995)
SBW25-*lacZ*	SBW25 carrying *′lacZ* marker in a phage locus	Zhang and Rainey (2007a)
PBR800	*hutF*::Ω or *PFLU0358*::Ω, insertion at nucleotide 423, Sp^R	Zhang and Rainey (2007b)
PBR1029	*hutF*::Ω SBW25::*hutRE*	This work
PBR1030	SBW25::*hutRE*	This work
Plasmids		
pCR8/GW/TOPO	Cloning vector, Sp^R	Invitrogen
pRK2013	Helper plasmid, Tra^+, Km^R	Ditta *et al*. (1980)
pUIC3	Integration vector with promoterless *′lacZ*, Mob^+, *ori*-R6K, Tc^R	Rainey (1999)
pUC18-mini-Tn*7*-Gm	Gm^r on mini-Tn*7*; for gene insertion in Gm^s bacteria	Choi and Schweizer (2006)
pUC18-mini-Tn*7*T-Gm-*lacZ*	*lacZ* transcriptional fusion vector; Gm^R on mini-Tn*7*T	Choi and Schweizer (2006)
pUX-BF13	Helper plasmid, Ap^R Mob^+ *ori*-R6K;	Choi and Schweizer (2006)

a. Gm, gentamicin; Km, kanamycin; Sp, spectinomycin; Tc, tetracycline.

rescens SBW25: 12.5 μg ml^{-1} tetracycline; 30 μg ml^{-1} kanamycin; 100 μg ml^{-1} ampicillin; 10 μg ml^{-1} gentamicin. For *P. aeruginosa* antibiotics were used at: 100 μg ml^{-1} tetracycline; 250 μg ml^{-1} carbenicillin; and 10 μg ml^{-1} gentamicin. *N*-formimino-L-glutamate was custom synthesized at > 95% purity by Dalton Pharma Services (Toronto, ON). *N*-formyl-L-glutamate was synthesized as described previously (Marti-Arbona *et al*., 2006).

Generation of unmarked deletion mutants

Unmarked gene deletions were constructed in *P. aeruginosa* PAO1 using a combination of gene splicing by overlap extension (SOE) PCR and two-step allelic exchange (Heckman and Pease, 2007). Briefly, oligonucleotide primers were used to amplify ~ 1000 bp nucleotide regions flanking the gene(s) of interest. A third PCR reaction was used to fuse the SOE flanking sequences together. The resulting ~ 2 kb deletion fragment was cloned into pCR8/GW/TOPO (Invitrogen). After the sequence of the cloned fragment was verified, the fragment was subcloned into the suicide vector pUIC3. Deletion constructs were introduced into *P. aeruginosa* PAO1 by tri-parental mating. Recipient *P. aeruginosa* strains were grown overnight at 43°C, then mixed with *E. coli* DH5α λpir carrying pUIC3, and *E. coli* carrying the helper plasmid pRK2013. Chromosomal integration of pUIC3 by a single homologous recombination event was selected by plating on LB agar containing tetracyline (100 μg ml^{-1}), nitrofurantoin (100 μg ml^{-1}) and X-gal (40 μg ml^{-1}). Nitrofurantoin was used to counterselect the *E. coli* DH5α λ_{pir} donor cells. After a period of non-selective growth in LB broth to allow the second homologous recombination event to take place, colonies were screened for tetracyline sensitivity, and clones carrying putative deletions were confirmed by PCR analysis and sequencing.

Competitive fitness assays

Cells taken from single colonies grown from −80°C stocks were pre-conditioned by cultivation for 24 h. Competitions were inoculated by mixing the two strains at a 1:1 volumetric ratio and incubated for 24 h. Initial and final competitor frequencies were determined by plating on LB agar plates supplemented with X-gal at 0 h and 24 h. To distinguish between competitor strains in each fitness assay, a mini-Tn*7* system was used to mark one of the competitors with a functional copy of *lacZ* (Choi and Schweizer, 2006). Control experiments showed that marking each strain had no effect on its fitness under the conditions used. Each relative fitness value is the mean of eight replicates and is expressed as the ratio of the Malthusian parameters (Lenski *et al*., 1991).

Construction of lacZ *promoter fusions and β-galactosidase assays*

Putative promoters were predicted using the Neural Network Promoter Prediction program (Reese, 2001). DNA fragments containing the predicted promoter region of the *hutRE* operon were amplified by PCR and cloned into pUC18-mini-Tn*7*T-Gm-*lacZ*. β-Galactosidase activities of the *′lacZ* reporter fusion strains were determined from cultures grown to mid-log phase in MMP, each supplemented with 20 mM of potential inducer. Assays were based on the hydrolysis of 4-methylumbelliferyl-β-D-galactoside to yield the fluorescent product, 7-hydroxy-4-methylcoumarin (4MU) as described

previously (Zhang and Rainey, 2007b). 4MU was detected at 460 nm after excitation at 365 nm using a Synergy 2 plate reader (BioTek Instruments). Each result is the mean and standard error of five independent cultures.

Phylogenetic analysis

The top 100 matches of a BLASTP search of the NCBI protein database (Sayers *et al*., 2010) for each gene of interest were downloaded and processed with a custom Perl script that extracted the species/strain name from the header and converted it into a NCBI taxonomy identification number to both avoid character limits and to allow leaf labelling by the iTOL server. Subsequently, the sequences were aligned with CLUSTALX (Larkin *et al*., 2007) and poorly aligned segments were removed with Gblocks (Castresana, 2000). The data set was then analysed with RAxML using the rapid bootstrap algorithm, with a WAG model with Γ-distributed rates (Stamatakis, 2006).

The *Pseudomonas* phylogenetic tree was inferred by maximum likelihood using TreePuzzle with a data set constructed by concatenating the 16S sequence [aligned in the RDP database (Cole *et al*., 2009)] with the *recA* and *gyrB* genes of fully sequenced members of the *Pseudomonas* genus, plus *Azotobacter vinelandii*, a species known to be nested within the genus *Pseudomonas* (Rediers *et al*., 2004; Young and Park, 2007). The resulting trees were then graphed with the iTOL server (Letunic and Bork, 2011), where the monophyletic taxa were manually collapsed with the exception of *Pseudomonas*. The resulting figures were manually coloured in Adobe Illustrator CS4.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The growth of *hut* pathway deletion mutants is indistinguishable from wild-type when grown in minimal medium P (with 20 mM glucose and ammonia). Growth of wild-type *P. aeruginosa* PAO1 (PBR1019, circles), a mutant with the *hutE* branch of the putative four-step pathway deleted (PBR1020, diamonds), a mutant with the *hutFG* branch of the five-step pathway deleted (PBR1021, squares) and a double mutant with both the *hutE* and *hutFG* branches deleted (PBR1022, triangles) was measured. Data are the means and standard errors calculated from three independent experiments; if not visible, the errors bars are contained within the symbol.

Fig. S2. *hutR* encodes a positive regulator.

A. Growth of the wild-type (PBR1019, circles), Δ*hutR* (PBR1026, triangles) and Δ*hutR*Δ*5* (PBR1028, diamonds) in minimal medium with glucose and ammonia as the carbon and nitrogen sources.

B. Growth of the wild-type, Δ*hutR* and Δ*hutR*Δ*5* in minimal medium with histidine as the sole carbon and nitrogen source. Without *hutR*, the strain cannot utilize the *hutE* branch of the four-step histidine utilization pathway.

Fig. S3. Phylogram of HutH (*P. aeruginosa* PAO1 gene locus PA5098, COG2989 group). Two groups appear in the phylogram, namely HutH sequences from the *Enterobacteriaceae* (in purple) and a group composed of HutH sequences from members of the *Pseudomonas* (in blue) with nested members of the *Cupriavidus*, *Achromobacter* and *Acinetobacter* genera (which are distantly related species). *Halomonas elongata* is believed to be basal to the genus *Pseudomonas* (Williams *et al*., 2010) as is seen in this tree of HutH (gene locus HELO_2475). The low support (16%) for HutH in *P. mendocina* (Pmen_4069) and its location contrary to the accepted lineage (Fig. 4) are indicative that it was incorrectly resolved in this analysis, but should be located within the group, containing the other fluorescent pseudomonads and the nested species, which has 84% support.

Fig. S4. Phylogram of HutU (Urocanase, PA5100, COG2987). The HutU phylogram is very similar to that of HutH (Fig. S2), where an enteric and a fluorescent pseudomonad group are seen, the latter with nested *Cupriavidus*, *Achromobacter* and *Acinetobacter* genera. The differences from Fig. S2 are that the *P. fluorescens* and *P. syringae* group is paraphyletic and not monophyletic, the location of the nested genera differs slightly, the *P. mendocina* HutU (Pmen_4070) falls basal to the *P. aeruginosa* HutU, following lineage, and HutU from *Halomonas elongata* (HELO_2475) is not present.

Fig. S5. Phylogram of HutI (imidazolone propionate amidohydrolase, PA5092, COG1228). Like the phylogram for HutU and HutH, the enteric and the expanded pseudomonad group are seen. However, the HutI protein sequence of members of *Achromobacter* and *Acinetobacter*, with the addition of *Burkholderia*, fall basal to those of the *Pseudomonas*. Despite the fact many species of *Burkholderia* were formerly classified as *Pseudomonas* species (group II pseudomonads), the genus is in a different class of Proteobacteria (Garrity, 2005). The HutI sequences of *Cupriavidus* species (RALTA_A2489 in *C. taiwanensis*) are distantly related, as is in *P. mendocina* (Pmen_4073) and in *H. elongata* (HELO_2478).

Fig. S6. Phylogram of HutF (forminoglutamate deiminase, PA5106, COG04026). As is seen with the phylogram of HutH, HutU and HutI, there is a group of protein from *Enterobacteriaceae* and a group composed of *Pseudomonas*, *Cupriavidus*, *Burkholderia* and some alphaproteobacterial species. The genus *Pseudomonas* resolves as a monophyletic group but the internal order is not correctly

resolved. *Pseudomonas mendocina* and *H. elongata* possess a four-step pathway only, i.e. they lack both hutF and hutG homologues.

Fig. S7. Phylogram of HutG (*N*-formylglutamate amidohydrolase, PA5091, COG3741). Like the phylogram of HutH, HutH, HutU, HutI and HutF there is an enteric group and an extended pseudomonad group, comprising *Cupriavidus* and *Burkholderia* with others species.

Fig. S8. Phylogram of HutC (regulator, PA5105, COG2188). Like in the phylogram of the five-step pathway members the two groups are seen. However, *A. vinelandii* DJ lacks a close relative of HutC. While in *P. mendocina* (Pmen_4068), *H. elongata* (HELO_2480), *Burkholderia* (BCEN_5906 BCEN_4641 in *B. cenocepacia*, the former in a *hutHCUIFG* operon) and *Marinomonas* species (Mmwyl1_3905 in strain MWYL1) the homologues are annotated as 'histidine utilization repressor' and are found in the operon with other hut genes but are not as closely related to *P. aeruginosa* HutC as the *Enterobacteriaceae*. In contrast to previous trees, *Acinetobacter* HutC (AXYL_00282 in *A. xylosoxidans*) is nested with the *Enterobacteriaceae*.

Fig. S9. Phylogram of HutD (regulator, PA5104, COG3758). Despite a much reduced number of enteric species possessing HutD, the two sister groups are seen in a similar way to the five-step pathway Hut enzymes and the HutC regulator. The genus *Pseudomonas* resolves monophyletically, albeit with incorrect internal order. Basal to this group are HutD genes from *Achromobacter* species (AXYL_00283 in *A. xylosoxidans*, same operon as HutC): this close relationship is also seen in HutC, HutH HutU and HutI (*Achromobacter* species have the four-step pathway). HutD from *Burkholderia* does not cluster close to the HutD of *Pseudomonas* species. In the same way as is seen for HutC, *A. vinelandii* lacks a close relative of HutD, while in *P. mendocina* (Pmen_4074 close to other *hut* genes), in *Burkholderia* (BCEN_5908 in *B. cenocepacia*) and in *Marinomonas* species (Mmwyl1_3904 in strain MWYL1, adjacent to *hutC*) are not as closely related to *P. aeruginosa* HutD as the *Enterobacteriaceae*.

Fig. S10. Phylogram of HutE (formiminoglutamase PA3175, COG0010). The HutE tree differs substantially from the other trees. Only *P. aeruginosa* strains, *P. fluorescens* Pf-5 and *P. mendocina* ymp possess a *hutE* gene. *Pseudomonas mendocina* HutE (Pmen_4072) does not cluster with those of other fluorescent pseudomonads, but with *Halomonas elongata* (HELO_2477) and other species. With the exception of *Providencia* species (purple), there are no *Enterobacteriaceae* representatives. Some *Ralstonia* species have two genes annotated as HutE (e.g. Rpic_2795 and Rpic_2879 in *R. pickettii*), hence the two clades in the figure. Unlike the phylograms of the sequences of the five-step pathway enzymes and the HutC and HutD regulators, the *P. aeruginosa* strains and the *P. fluorescens* Pf-5 strain cluster with 'Aromatoleum aromaticum' (EBA5739) and *Pseudoalteromonas atlantica* (Palt_1018), which are not closely related to *Pseudomonas*: the former is a betaproteobacterium, whereas *P. atlantica* is a former pseudomonad now classified in a different order of the *Gammaproteobacteria* from that of *Pseudomonas* species (Williams *et al*., 2010).

Fig. S11. Phylogram of HutR (regulator of *hutE*, PA3174, COG1414). The tree of HutR is different from that of HutE, but shares many more similarities to HutE in the vicinity of the *P. aeruginosa* strains and the *P. fluorescens* Pf-5 strain, than to the trees from the peptide sequences of the other *hut* genes. In fact in *Achromobacter*, *Cupriavidus*, *Ralstonia*, *Burkholderia* and *Bordetella*, which are species with similar relations in both the HutE and HutR trees, the *hutE* and *hutR* genes are close to each other in the genome (data not shown).

Fig. S12. The *hutRE* module is functional when transferred to *P. fluorescens* SBW25.

A. Growth of SBW25 strains in minimal medium with histidine as the sole carbon and nitrogen source. Addition of the *hutRE* module ($^{+4}$) restores growth to a strain with the five-step pathway inactivated (*hutF*::Ω) demonstrating that *hutRE* is functional when it is transferred to SBW25.

B. Competitive fitness of SBW25^{+4} (SBW25::*hutRE*; both branches present) in competition with a *lacZ*-marked 'wild-type' strain (SBW25-*lacZ*). Values less than 1 (dashed line) indicate a lower relative fitness of the SBW25^{+4} relative to wild-type. Key: median (horizontal lines in boxes), interquartile range (boxes), 90th and 10th percentiles (vertical bars), values with significant deviation from 1 ($P < 0.05$ for a two-tailed test with the null hypothesis that $W = 1$, calculated using the t distribution and 7 d.f.) are indicated (*).

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