Pseudomonas aeruginosa PAO1 genes for 3-guanidinopropionate and 4-guanidinobutyrate utilization may be derived from a common ancestor

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Pseudomonas aeruginosa PAO1 utilizes 3-guanidinopropionate (3-GP) and 4-guanidinobutyrate (4-GB), which differ in one methylene group only, via distinct enzymes: guanidinopropionase (EC 3.5.3.17; the *gpuA* product) and guanidinobutyrase (EC 3.5.3.7; the *gbuA* product). The authors cloned and characterized the contiguous *gpuPAR* genes (in that order) responsible for 3-GP utilization, and compared the deduced sequences of their putative protein products, and the potential regulatory mechanisms of *gpuPA*, with those of the corresponding *gbu* genes encoding the 4-GB catabolic system. GpuA and GpuR have similarity to GbuA (49% identity) and GbuR (a transcription activator of *gbuA*; 37% identity), respectively. GpuP resembles PA1418 (58% identity), which is a putative membrane protein encoded by a potential gene downstream of *gbuA*. These features of the GpuR and GpuP sequences, and the impaired growth of *gpuR* and *gpuP* knockout mutants on 3-GP, support the notion that GpuR and GpuP direct the 3-GP-inducible expression of *gpuA*, and the uptake of 3-GP, respectively. Northern blots of mRNA from 3-GP-induced PAO1 cells revealed three transcripts of *gpuA*, *gpuP*, and *gpuP* and *gpuA* together, suggesting that *gpuP* and *gpuA* each have a 3-GP-responsible promoter, and that some transcription from the *gpuP* promoter is terminated after *gpuP*, or proceeds into *gpuA*. Knockout of *gpuR* abolished 3-GP-dependent synthesis of the transcripts, confirming that GpuR activates transcription from these promoters, with 3-GP as a specific co-inducer. The sequence conservation between the three functional pairs of the Gpu and Gbu proteins, and the absence of *gpuAPR* in closely related species, imply that the triad *gpu* genes have co-ordinately evolved from origins common to the *gbu* counterparts, to establish an independent catabolic system of 3-GP in *P. aeruginosa*.

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INTRODUCTION

Unlike many pseudomonads that utilize 4-guanidinobutyrate (4-GB), but not 3-guanidinopropionate (3-GP), as the carbon and nitrogen source, *Pseudomonas aeruginosa* can use either (Tricot *et al.*, 1990; Nakada & Itoh, 2002; Yorifuji *et al.*, 1983). The catabolic route of 4-GB in *Pseudomonas putida* and *P. aeruginosa* is integrated into the arginine dehydrogenase (ADH) pathway of arginine catabolism (Chou & Rodwell, 1972; Cunin *et al.*, 1986; Haas *et al.*, 1990; Jann *et al.*, 1988; Vanderbilt *et al.*, 1975) (Fig. 1). This pathway of *P. putida* starts with the oxidative deamination of L-arginine by ADH (also called L-arginine oxidase) to 2-ketoarginine, which is decarboxylated to 4-guanidinobutyraldehyde. The oxidation product of 4-guanidinobutyraldehyde, 4-GB, is converted by guanidinobutyrase (EC

3.5.3.7; the *gbuA* product) into 4-aminobutyrate, before being channelled into the tricarboxylic acid cycle (Fig. 1). *P. aeruginosa* PAO1 also has all of the ADH pathway enzymes, except for ADH itself. Racemase and D-arginine dehydrogenase are thought to convert L-arginine to 2-ketoarginine via the D-isomer in this bacterium (Fig. 1). Exogenous 2-ketoarginine and its downstream intermediates, including 4-GB, are effective carbon and nitrogen sources for this strain (Haas *et al.*, 1990; Jann *et al.*, 1988; Nakada & Itoh, 2002).

The *P. aeruginosa* PAO1 GbuA has been purified and characterized in some detail (Nakada & Itoh, 2002; Yorifuji & Sugai, 1978). This enzyme belongs to the arginase/agmatinase family of proteins, and is highly specific to 4-GB. Guanidinopropionase (EC 3.5.3.17) has also been purified from this strain (Yorifuji & Sugai, 1978; Yorifuji *et al.*, 1982, 1983). We refer to the gene for this enzyme as *gpuA*. GpuA catalyses the specific hydrolysis of 3-GP into β -alanine and urea (Yorifuji & Sugai, 1978); the former product can be

Abbreviations: ABS, activation binding site; ADH, arginine dehydrogenase; 4-GB, 4-guanidinobutyrate; 3-GP, 3-guanidinopropionate; RBS, recognition binding site.

Fig. 1. Catabolism of 3-GP and 4-GB in *P. aeruginosa* PAO1. Only relevant intermediates and genes are presented. The *gpuA* and *gbuA* genes encode guanidinopropionase and guanidinobutyrase, respectively.

used as a source of carbon and nitrogen, and as a precursor for the synthesis of fatty acids, amino acids and other metabolites, after conversion into acetyl-CoA (Fig. 1). The strict substrate specificity of both GbuA and GpuA has allowed the isolation of *gbuA9005* (defective in 4-GB utilization) and *gpu-9018* (defective in 3-GP utilization) mutants (Haas *et al.*, 1984). We cloned and characterized *gbuA*, along with the cognate *gbuR* regulator gene, which encodes a regulatory protein that directs the inducible expression of *gbuA* in the presence of exogenous 4-GB (Nakada & Itoh, 2002). The *gpu-9018* locus has been mapped at 18 min of the chromosome, but not yet cloned (Haas *et al.*, 1984; Holloway *et al.*, 1994). Although GbuA and GpuA use different substrates with high specificity, both enzymes act on a scissile guanidino-bond, and they have a Mn^{2+} requirement for activity, a high optimum pH, and similarly sized native forms and subunits (Nakada & Itoh, 2002; Yorifuji *et al.*, 1982). Furthermore, peptide maps of the enzymes have suggested some similarity in their amino acid sequences (Yorifuji *et al.*, 1983).

To examine the molecular structure of GpuA, and the regulatory mechanism underlying the specific induction of GpuA synthesis by exogenous 3-GP, we cloned the *gpu-9018* locus, and identified three genes (*gpuPAR*) that are required for 3-GP utilization. Structural and functional analyses of these genes established that *gpuA* is an allele of *gpu-9018*, that *gpuP* encodes a 3-GP transport protein having homology to PA1418 (a gene downstream of *gbuA*), and that *gpuR* specifies a transcription activator of *gpuPA*. The striking sequence similarity between the Gpu and Gbu counterparts implies that the *gpu* and *gbu* gene trios have evolved from common triad ancestors.

METHODS

Strains, plasmids and media. *P. aeruginosa* PAO strains and *Escherichia coli* (Table 1) were cultured in nutrient yeast broth (NYB) or Luria-Bertani (LB) medium, respectively, supplemented with antibiotics when necessary (Haas *et al.*, 1977; Hoang *et al.*, 1998; Sambrook *et al.*, 1989). The *P. aeruginosa* PAO strains were grown in minimal medium P (MMP; Haas *et al.*, 1977) containing the indicated carbon and nitrogen sources at a concentration of 20 mM for strain construction, growth experiments and enzyme assays.

DNA techniques. DNA purification, restriction enzyme analysis, and other DNA techniques were carried out as described (Nakada & Itoh, 2002; Nishijyo *et al.*, 2001; Sambrook *et al.*, 1989). Nucleotide sequences on both strands were determined using an ABI310 DNA sequencer (Perkin-Elmer) and ABI Big-dye Terminator Cycle sequencing kits (Perkin-Elmer).

Cloning the *gpu-9018* locus. A plasmid library of *Sau3A*I fragments of the PAO1 chromosomal DNA was constructed in *E. coli* XL-1 Blue by shotgun cloning into the mobilizable shuttle vector pNIC6011 (Nishijyo *et al.*, 2001), and library plasmids were then transferred into strain PAO4173 (*gpu-9018*) by conjugation using the helper *E. coli* HB101/pRK2013 (Comai *et al.*, 1983), followed by selection of 3-GP-utilizing (Gpu^+) transconjugants on MMP agar containing 125 μ g carbenicillin ml^{-1} , and 20 mM 3-GP as the carbon and nitrogen source. After restriction analysis, and sequencing the plasmid inserts in several Gpu^+ transconjugants, we further investigated plasmid pYJ104 carrying a 4.5 kb *gpu-9018* fragment.

Plasmid and strain construction. To localize *gpu-9018* on the chromosomal DNA region cloned in plasmid pYJ104 by complementation tests, we constructed deletion and insertion derivatives as follows. Removal of the 1.0 kb *Bgl*II-*Hind*III fragment carrying portions of PA289 and PA290 yielded plasmid pYI1035 (Fig. 2b). Insertion of a gentamicin-resistant (Gm) cassette from plasmid pPS858 (Hoang *et al.*, 1998) into the *Pvu*II site of PA288 as a *Sma*I fragment, and into the *Bam*HI site of PA287, as a *Bam*HI fragment, created plasmids pYI1041 and pYI1043, respectively (Fig. 2b). Similarly, insertion of an Ω Sp/Sm interposon (Fellay *et al.*, 1987) into the same sites of PA288 and PA287 resulted in plasmids pYI1047 and pYI1057, respectively (Fig. 2c). Insertion of the interposon into the *Bgl*II site in the 3' region of PA286 generated plasmid pYI1060 (Fig. 2c). To construct knockout mutants of PA287, PA288 and PA289, appropriate DNA regions carrying the genes of interest were cloned into the suicide plasmid pEX18Ap (*mob*⁺ *sacB*) (Hoang *et al.*, 1998), followed by the insertion of a Gm cassette (Hoang *et al.*, 1998) into the *Bam*HI, *Pvu*II and *Sma*I sites of PA287, PA288 and PA289 on the resultant plasmids, respectively. These constructs were then conjugated into strain PAO1 (Nishijyo *et al.*, 2001), and Gm^R transconjugants harbouring the plasmid sequences integrated at the corresponding chromosome locus by recombination were selected on LB agar containing gentamicin (100 μ g ml^{-1}). Subsequent selection on LB containing 5% (w/v) sucrose, which prevents the growth of cells having the pEX18Ap (*sacB*) sequence (Hoang *et al.*, 1998), yielded strains PAO4520 (PA289 = *gpuR*::Gm), PAO4522 (PA288 = *gpuA*::Gm) and PAO4524 (PA287 = *gpuP*::Gm). Correct insertion of the Gm cassette and Ω Sp/Sm at the relevant sites was verified by PCR (Nishijyo *et al.*, 2001).

Northern blotting, primer extension and RT-PCR. Total RNA was isolated from PAO1 cells exponentially growing (OD₆₀₀ 0.3) in MMP containing the indicated carbon and nitrogen sources at 20 mM, as described (Nishijyo *et al.*, 2001). Samples of RNA (50 μ g) in 10% (w/v) glyoxal were resolved, together with RNA markers (Toyobo Biochemicals), on 1.0% (w/v) agarose HS (Nippon Gene), and blotted onto Hybond-N+ nylon membranes

Table 1. Strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
Strains		
<i>P. aeruginosa</i>		
PAO1	Wild-type	Stover <i>et al.</i> (2000)
PAO4173	<i>gbuA9005 gpu-9018</i>	Nakada & Itoh (2002)
PAO4520	<i>gpuR::Gm</i>	This study
PAO4522	<i>gpuA::Gm</i>	This study
PAO4524	<i>gpuP::Gm</i>	This study
<i>E. coli</i>		
DH5 α	F ⁻ /endA1 <i>hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44 thi-1 recA1 gyrA relA1</i> Δ (<i>lacIZYA-argF</i>)U169 <i>deoR</i> [ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15]	Bethesda Research Laboratories
HB101	<i>supE44 hsdS20</i> (r _B ⁻ m _B ⁻) <i>recA13 ara-14 proA2 lacY galK2 rpsL20 xyl-5 mtl-1</i>	Sambrook <i>et al.</i> (1989)
XL-1 Blue	<i>endA1 gyrA96 thi-1 hsdR17 supE44 recA1 lac</i> [F', <i>proAB lac</i> ^R Z Δ M15 Tn10 (Tc ^R)]	Stratagene
Plasmids		
pEX18Ap	Ap ^R , ColE1 replicon, <i>oriT sacB</i>	Hoang <i>et al.</i> (1998)
pRK2013	Km ^R , ColE1 replicon, <i>tra</i> (RK2)	Comai <i>et al.</i> (1983)
pPS858	Ap ^R , Gm ^R , ColE1 replicon carrying a Gm cassette	Hoang <i>et al.</i> (1998)
pUC118	Ap ^R , ColE1 replicon	Vieira & Messing (1987)

(Amersham Pharmacia Biotech) using a GenVac Blotter (Pharmacia LKB). Transcripts of *gpuAPR* blotted on the membranes were hybridized with a *gpuA* probe (a *PvuII*–*BstEII* fragment of 421 bp; nt +380 to +800), or a *gpuP* probe (a 921 bp *EcoO109I*–*SmaI* fragment; nt –81 to +840) labelled with [α -³²P]dCTP (220 TBq mmol⁻¹; Amersham Pharmacia Biotech). The hybridized DNA fragments on the membranes were detected by exposure to X-ray film. In primer extension experiments, RNA samples (20 μ g) were annealed with oligonucleotides (5'-GCGCATGAAGTCGGGATACCGGCGAAG-3', complementary to the region at nt +48 to +75 of *gpuA*; and 5'-CGAGGAAATCGCTCTGGCTCTTCGCCTTG-3', complementary to the region at nt +72 to +100 of *gpuP*), and end-labelled with [γ -³²P]ATP (220 TBq mmol⁻¹; Amersham Pharmacia Biotech) using polynucleotide kinase (Takara Bio). A complementary strand was synthesized using avian reverse transcriptase (RAV-2; Takara Bio) in the presence of deoxyribonucleotides. Synthesized cDNAs were then resolved on 6% (w/v) denatured polyacrylamide gels, along with sequence ladders generated using the BcaBEST sequencing kit (Takara Bio), the ³²P-end-labelled oligonucleotide primers, and plasmid pYI104 as the template. Radioactive DNA fragments on the gels were visualized on X-ray film. We used a BcaBEST RNA PCR kit (Takara Bio) to confirm read-through of the transcription from the *gpuP* promoter into *gpuA*, and to determine relative amounts of *gpuA* and *gpuP* transcripts by RT-PCR. cDNAs of *gpuP* and *gpuA* mRNA regions were synthesized using the above total RNA samples, and oligonucleotides complementary to +933 to +957 of *gpuA* (primer A1), and +664 to +692 of *gpuP* (primer P1), respectively, under the conditions recommended by the supplier; primer A1 forms both *gpuA* and *gpuPA* cDNAs (*gpuA/PA* cDNA), and primer P1 forms *gpuP* cDNAs. Amplification of various regions by PCR (denaturing at 94 °C for 30 s, annealing at 57 °C for 30 s, and elongation at 72 °C for 1 min; 28 cycles) proceeded using the following cDNAs and oligonucleotide pairs: *gpuA/PA* cDNA with primers A1 and A2 (nt +273 to +302 of *gpuA*) for *gpuA* cDNA; *gpuP* cDNA with primers P1 and P2 (corresponding to nt –37 to –9 of *gpuP*) for *gpuP* cDNA; and *gpuA/PA* cDNA with

primers PA1 (nt +992 to +1020 of *gpuP*) and PA2 (complementary to +312 to +340 of *gpuA*) for the intergenic region between *gpuP* and *gpuA*. The PCR products were resolved on 1.0% agarose gels by electrophoresis, and stained with ethidium bromide.

Enzyme assays. We prepared extracts from cells growing exponentially (OD₆₀₀ 0.5) in MMP containing L-glutamate (a control), 3-GP, L-glutamate and 3-GP, or 4-GB, as sole carbon and nitrogen sources (each at 20 mM), as described (Nakada & Itoh, 2002). Guanidinopropionase was assayed (in triplicate) under the same conditions as guanidinobutyrase (Nakada & Itoh, 2002), except the pH of the glycine buffer was 9.0 instead of 9.5. The reaction product, urea, was measured according to Chou & Rodwell (1972). One enzyme unit was defined as the amount of enzyme required to form 1 μ mol product min⁻¹. Protein concentrations were determined using a Protein Assay Kit (Bio-Rad), with BSA as the standard.

RESULTS

Cloning and structure of the *gpuAPR* genes

We cloned a 4.5 kb *Sau3AI* fragment carrying the *gpu-9018* locus into plasmid pYI104 (Fig. 2b) by functional complementation of the 3-GP-utilization (Gpu⁺) phenotype of strain PAO4173 (*gpu-9018*), as described in Methods. Nucleotide sequencing revealed that this insert corresponded to nt 321 848 to 326 320 of the PAO1 genome, which contain three complete and two truncated genes of suggested or unknown function (<http://www.pseudomonas.com>) (Fig. 2a, b). The first gene was the 3' portion of PA286, encoding a probable fatty acid desaturase with 85% similarity to the stearyl-CoA desaturase of *Azotobacter vinelandii* (Mylona *et al.*, 1996). The second gene (PA287) encoded a possible transport protein (461 aa) of *M_r* 48 461

Fig. 2. Gene organization of (a) the *gpuPAR* locus, and knockout mutants of the *gpu* genes, (b) plasmids used in complementation tests, and (c) plasmids used in analysis of *gpuPAR* transcription *in vivo*. (a) Arrows below the map indicate transcripts of *gpuPAR* genes. (b) Plasmids were derived from plasmid pYJ104 by deletion or by insertion of a Gm cassette (Hoang *et al.*, 1998) at the indicated restriction sites. Complementation tests were carried out on MMP agar (Haas *et al.*, 1977) supplemented with 20 mM 3-GP as the source of carbon and nitrogen. +, Growth of single colony within 18 h; -, no growth at 48 h. (c) Mutant plasmids were constructed by insertion of a ΩSp/Sm interposon carrying a transcription terminator (Fellay *et al.*, 1987) at the indicated restriction sites of the target genes. Abbreviations for restriction sites: Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; Pv, *Pvu*II; Sm, *Sma*I; Su, *Sau*3AI (only sites used in construction of pYJ104 are indicated); X, *Xho*I.

with 12 potential transmembrane helices, and 46 % similarity to the PanF sodium/pantothenate symporter of *E. coli* (Jackowski & Alix, 1990). The product of this gene had 58 % identity to the product of PA1418 located downstream of *gbuA* (Nakada & Itoh, 2002). The third gene (PA288) specified a putative amidinohydrolase (371 aa, M_r 38 859) that shares 57 % similarity with *E. coli* agmatinase (the *speB* product), a putrescine biosynthetic enzyme (Glansdorff, 1996), and hence called *speB1* (<http://www.pseudomonas.com>). The fourth gene (PA289) specified a putative regulatory protein (297 aa, M_r 33 385) homologous (<40 % identity) to the LysR family of proteins (Schell, 1993), including GbuR (37 % identity). The fifth, and last, truncated

gene (PA290) on the insert encoded a polypeptide (232 aa) of M_r 36 387 that was homologous to the PleD protein of *Caulobacter crescentus* (Hecht & Newton, 1995). Knockout of PA287 and PA289, as well as PA288 (*speB1*), resulted in impaired growth on 3-GP (see below), suggesting that these genes are important for 3-GP utilization. We therefore designated PA287, PA288 and PA289 as *gpuP*, *gpuA* and *gpuR*, respectively (Fig. 2a).

Identification of *gpu-9018* as *gpuA*

To localize *gpu-9018* on the cloned DNA fragment, we constructed deletion and insertion derivatives from plasmid

pYJ104, and tested their ability to restore the Gpu⁺ phenotype of strain PAO4173 (*gpu-9018*). Plasmid pYI1035, having a deletion in the 3' portion of *gpuR*, restored the Gpu⁺ phenotype of the mutant, whereas plasmid pYI1041, having an insertion of a Gm cassette in *gpuA*, did not (Fig. 2b). Moreover, inactivation of *gpuA* on the chromosome by insertion of the Gm cassette, as in strain PAO4522 (Fig. 2b), abolished GpuA synthesis, resulting in the Gpu⁻ phenotype (see below). These results, and the similarity of the molecular mass between the deduced GpuA (*M_r* 38 869) and purified guanidinopropionase (35 kDa) (Yorifuji *et al.*, 1982), supported the notion that *gpuA* is an allele of *gpu-9018*, and specifies guanidinopropionase. We accordingly renamed *gpu-9018* as *gpuA9018*. Sequencing of *gpuA9018* identified a transversion of T at nt 168 to G, which caused an alteration of His at amino acid position 56 to Gln.

Functions of *gpuP* and *gpuR* in 3-GP utilization

Strain PAO1 did not form a measurable amount [<0.1 units (mg protein)⁻¹] of GpuA during growth in MMP containing either L-glutamate or 4-GB. When 3-GP was present, this strain produced the enzyme in quantities of up to 1.4 ± 0.1 units (mg protein)⁻¹. When presented together with 3-GP, L-glutamate minimally affected GpuA synthesis [1.2 ± 0.1 units (mg protein)⁻¹]. To determine whether *gpuP* and *gpuR* are involved in 3-GP utilization, and affect GpuA synthesis, we constructed knockout mutants PAO4524 (*gpuP*::Gm) and PAO4520 (*gpuR*::Gm) by inserting a Gm cassette (Hoang *et al.*, 1998) into the *Bam*HI site of *gpuP*, and into the *Sma*I site of *gpuR*, respectively, on the chromosome of strain PAO1 (Fig. 2a). Strains PAO4524 (*gpuP*::Gm) and PAO4520 (*gpuR*::Gm) did not grow on MMP agar containing 3-GP as the sole source of carbon and nitrogen. The amounts of GpuA in these knockout mutants cultured in MMP containing L-glutamate and 3-GP were negligible [<0.1 units (mg protein)⁻¹]. The observed Gpu⁻ phenotype of the mutants thus appeared to correlate with the inability to express GpuA, probably as a consequence of impaired uptake of the inducer 3-GP (in strain PAO4524), and of the absence of the GpuR transcription activator for the enzyme gene (in strain PAO4520).

Transcription units of the *gpuPAR* genes

The *gpuPAR* genes are all located in the same orientation, with intergenic spaces for promoters: 230 bp between *PA286* and *gpuP*, 56 bp between *gpuP* and *gpuA*, and 53 bp between *gpuA* and *gpuR* (Fig. 2a). We initially analysed transcription units of *gpuPAR* *in vivo* using plasmids with an inserted Ω interposon carrying a transcriptional terminator (Fellay *et al.*, 1987) on *PA286*, *gpuP* or *gpuA* (Fig. 2c). Plasmids pYI1047 (*gpuA*:: Ω Sp/*Sm*), pYI1057 (*gpuP*:: Ω Sp/*Sm*) and pYI1060 (*PA286*:: Ω Sp/*Sm*) (Fig. 2c) each restored the Gpu⁺ phenotype of strains PAO4520 (*gpuR*::Gm), PAO4522 (*gpuA*::Gm) and PAO4524 (*gpuP*::Gm), respectively, indicating that the *gpuPAR* genes each have their own promoter (Fig. 2a).

Transcription from the predicted *gpuA* (*P_{gpuA}*), *gpuP* (*P_{gpuP}*) and *gpuR* (*P_{gpuR}*) promoters was further investigated by Northern blotting, using RNA samples prepared from PAO1 cells cultured in MMP containing a non-inducible substrate, glutamate or 4-GB, or the inducible substrate 3-GP, and from PAO4520 (*gpuR*::Gm) cells cultured in MMP containing both L-glutamate and 3-GP (inducible conditions). No *gpuP* and *gpuA* transcripts were detected in RNA samples from cells grown in L-glutamate or 4-GB medium (Fig. 3, lanes 1, 2, 4 and 5), or from the *gpuR* mutant cells (data not shown). In contrast, 1300 and 2400 nt transcripts were detected with a *gpuA* probe (+380 to +800 of *gpuA*) in the RNA sample from the cells with induced 3-GP (Fig. 3, lane 3) at a ratio of 7.6:1. Probing with a *gpuP* sequence (nt -81 to +840 of *gpuP*) detected 2400 and 1500 nt transcripts in the same RNA sample (Fig. 3, lane 6). These results support the notion that *P_{gpuP}* and *P_{gpuA}* exist, and suggest that some transcription from *P_{gpuP}* proceeds into *gpuA*.

We quantified the *gpuP* and *gpuA* transcripts, and investigated the possible read-through of transcription from *P_{gpuP}* into *gpuA* by RT-PCR. We synthesized cDNAs for *gpuA*, *gpuP* and *gpuPA* mRNAs using as templates total RNAs extracted from PAO1 cells induced or uninduced with 3-GP, and oligonucleotides complementary to appropriate sites of the genes as primers (see Methods). Specific regions of the cDNAs were subsequently amplified by PCR using the cDNA samples and pairs of oligonucleotides corresponding to the sites of interest. Primers A1 and A2 amplified 685 bp fragments of *gpuA* (nt +273 to +957) from the *gpuA/PA* cDNAs (Fig. 4, lane 2), and primers P1 and P2 synthesized 729 bp fragments of *gpuP* (nt -37 to +692) from the *gpuP* cDNA (Fig. 4, lane 4). Fragments were not amplified when the cDNAs were synthesized from the uninduced cells

Fig. 3. Northern blots of *gpuPA* transcripts. RNA samples prepared from PAO1 cells grown in MMP plus 20 mM glutamate (lanes 1 and 4), MMP plus 20 mM 4-GB (lanes 2 and 5), and MMP plus 20 mM 3-GP (lanes 3 and 6), were resolved on agarose gels and blotted onto Hybond-N+ nylon membranes. Transcripts were detected using ³²P-labelled probes, *gpuA* (nt +380 to +800 from translation initiation codon) (lanes 1–3) and *gpuP* (nt -81 to +840) (lanes 4–6). Numbers left and right of the gel indicate sizes of RNA markers and transcripts, respectively.

Fig. 4. RT-PCR analyses of *gpuA* and *gpuP* transcripts and intergenic region. cDNAs for *gpuA* and *gpuPA* mRNAs (*gpuA/PA* cDNAs upstream from nt +957 of *gpuA*), and for *gpuP* mRNA (*gpuP* cDNA upstream from nt +692 of *gpuP*), were synthesized using total RNA extracted from PAO1 cells cultured in MMP containing L-glutamate or 3-GP, and primer A1 or primer P1, respectively. The cDNAs and oligonucleotide primers used in PCR were: lanes 1 and 2, *gpuA/PA* cDNAs and primers A1 and A2; lanes 3 and 4, *gpuP* cDNA and primers P1 and P2; lanes 5 and 6, *gpuA/PA* cDNAs and primers PA1 and PA2. Lanes 1, 3 and 5, cDNAs synthesized with RNA samples from uninduced cells; lanes 2, 4 and 6, cDNAs from cells with induced 3-GP. Primer sequences: primer A1, complementary to nt +933 to +957 of *gpuA*; primer A2, nt +273 to +302 of *gpuA*; primer P1, complementary to nt +664 to +692 of *gpuP*; primer P2, nt -37 to -9 of *gpuP*; primer PA1, nt +992 to +1020 of *gpuP*; primer PA2, complementary to nt +312 to +340 of *gpuA*. M, *EcoRI* and *HindIII* digests of λ DNA as size markers.

(Fig. 4, lanes 1, 3 and 5). The ratio of the amplified *gpuA* and *gpuP* fragments was about 7:1, in accordance with the relative amounts of transcripts generated from P_{gpuP} and P_{gluA} (Fig. 3). A 789 bp fragment (nt +992 of *gpuP* to +340 of *gpuA*; Fig. 4, lane 6) that included the 56 bp intergenic spacer between the two genes was amplified by PCR from the *gpuA/PA* cDNA samples using primers PA1 and PA2, confirming that some transcription from P_{gpuP} proceeds into *gpuA*.

Identification of the P_{gpuA} and P_{gpuP} promoters

To localize the *gpuA*, *gpuP* and *gpuR* promoters, we determined the 5' ends of their transcripts by primer extension using RNA samples from PAO1 cells cultured in MMP with L-glutamate or 3-GP. The cDNAs of these genes synthesized from the uninduced cells grown in L-glutamate were not detected. On the other hand, an RNA sample from the 3-GP-induced cells yielded two cDNAs for the *gpuA* transcript,

and one for the *gpuP* transcript (Fig. 5a, and b). However, a cDNA for the *gpuR* transcript was not detected (data not shown), indicating that the expression level of this regulatory gene was very low. A comparison with sequence ladders localized the 5' ends of the *gpuA* transcripts at 296 and 299 bp upstream from the translation initiation ATG codon of *gpuA* (Fig. 5a), allowing identification of the -35 (5'-TGGTCA-3') and -10 (5'-GAGCAT-5') sequences of P_{gpuA} at around 1080 nt of the *gpuP* coding region (1386 nt). The 5' end of the *gpuP* transcript was located 53 bp upstream of the translation initiation ATG codon of this gene (Fig. 5b), positioning the -35 (5'-TTCAGC-3') and -10 (5'-TATCTT-3') sequences of P_{gpuP} at appropriate distances from the 5' end. The -10 and -35 sequences of these promoters resembled those for the σ^{70} RNA polymerase holoenzyme.

DISCUSSION

Peptide mapping has revealed some similarity between the GpuA and GbuA sequences (Yorifuji *et al.*, 1983) that can now be examined at the amino acid level. These enzymes of the arginase/agmatinase family share 49 % identity and 68 % similarity in terms of amino acids. Because of their similarity of about 57 % to *E. coli* agmatinase (the *speB* product) involved in putrescine synthesis (Glansdorff, 1996), the genes of GpuA and GbuA have been annotated as *speB1* and *speB2*, respectively (<http://www.pseudomonas.com>). The absence of any other *speB* homologues in *P. aeruginosa* PAO1 indicates that this strain has no *speB*. In fact, this strain recruits agmatine deiminase (the *aguA* product) and N-carbamoylputrescine amidohydrolase (the *aguB* product), which were discovered as the catabolic enzymes of agmatine (Nakada *et al.*, 2001) in the biosynthetic conversion of agmatine to putrescine (Nakada & Itoh, 2003). A detailed phylogenetic analysis (Sekowska *et al.*, 2000) has divided the arginase/agmatinase family of proteins into arginases, agmatinases, and a third group of plant and bacterial enzymes, including the SpeB1 and SpeB2 of *P. aeruginosa* PAO1. Our previous identification of SpeB2 as GbuA assumed that the third group consists of amidinohydrolases that act upon 4-GB or other guanidino acids (Nakada & Itoh, 2002). The present identification of SpeB1 as GpuA supports this assumption.

The *PA1418* product has apparent similarity (58 % identity) to GpuP, and this gene is located near *gbuAR* (Nakada & Itoh, 2002), implying its involvement in 4-GB uptake. However, *PA1418* knockout minimally affects the Gbu⁺ phenotype (Nakada & Itoh, 2002). Thus, *P. aeruginosa* PAO1 must have a 4-GB transport gene that is not linked to *gbuAR*. If *PA1418* encodes a 4-GB transport protein, a second transport gene would hinder the effect of the *PA1418* mutation on 4-GB utilization. Therefore, a possible role of *PA1418* in 4-GB transport cannot be ruled out at this stage. To clarify the role of *PA1418* in 4-GB import, the second transport gene should be identified.

Fig. 5. Primer extension analysis of *gpuA* (a) and *gpuP* (b) transcripts. Complementary strands were synthesized using ^{32}P -end-labelled oligonucleotides (corresponding to nt +48 to +75 of *gpuA*, and to nt +72 to +100 of *gpuP*) as primers and RNA samples from PAO1 cells grown in MMP plus 20 mM glutamate (lane 1), or MMP plus 20 mM 3-GP (lane 2), and then resolved on 6% (w/v) denatured polyacrylamide gels, together with sequence ladders (G, A, T and C). Asterisks indicate 5' ends of *gpuA* and *gpuP* transcripts.

Proteins of the LysR family, to which GpuR and GbuR belong, have a helix–turn–helix DNA-binding motif of about 20 aa at the N terminus, a coinducer recognition domain at the centre, and a C-terminal domain important for both DNA binding and the coinducer response (Schell, 1993). Helix–turn–helix motifs have been located in both GpuR (aa 28–48) and GbuR (aa 22–41) at the N termini, using a helix–turn–helix program (Dodd & Egan, 1990). These motifs are relatively well conserved, sharing 50% identical residues. The possible coinducer-binding domains (aa 96–177 of GpuR, and aa 90–171 of GbuR) are slightly more homologous (42% identity) than the overall amino acid identity (37%) of the proteins. However, frequent substitutions must have arisen to match the coinducer-binding domains with specific coinducers that differ in one methylene group only. Upon interaction with a coinducer, LysR proteins bind to two regulatory sites around the –70 (recognition binding site; RBS) and –35 (activation binding site; ABS) regions of promoters, allowing the regulatory proteins to interact with the α subunit of the RNA polymerase holoenzyme (McFall *et al.*, 1998). The core 10 residues (F/LSGT/GYIGYIP) of the C-terminal domain (corresponding aa 241–250 of GpuR) are very similar between GpuR and GbuR. The RBS and ABS motifs for the LysR family proteins have characteristic hairpin structures with a stem (3–6 bp) and a loop (5–7 bp) (Schell, 1993). Possible RBS and ABS motifs can be found around the –80 and –35 regions, respectively, of the *gpuA* and *gpuP* promoters and of the *gbuA* promoter (Nakada & Itoh 2002). Binding experiments are warranted to demonstrate the functions of these putative motifs in the inducible expression of the genes for GpuR and GbuR.

The cellular concentrations of GpuP and GpuA required to accomplish their roles appear to be controlled at the level of transcription (Figs 3 and 4). About half of the transcription from the *gpuP* promoter terminates after *gpuP* (Figs 2a and 3), probably due to the terminator-like structure of the 9 bp stem and 3 bp loop [ΔG –26.3 kcal mol $^{-1}$ (–110.04 kJ mol $^{-1}$)] located 8 bp downstream of the translation termination codon, but the other half proceeds into *gpuA* over the termination signal for transcription (Figs 2a and 3). Primer extension experiments localized the

–35 and –10 sequence of the *gpuA* promoter within the *gpuP*-coding region, and transcription from this promoter starts before the termination signal. However, because of the powerful transcriptional activity of the *gpuA* promoter, and the weak attenuation of transcription by the terminator, transcription from the *gpuP* promoter can proceed into *gpuA* beyond the termination signal.

The most striking features of the 3-GP and 4-GB utilization components are that GpuP, GpuA and GpuR have significant homology to PA1418, GbuA and GbuR, respectively. Although a role for PA1418 in 4-GB uptake remains to be demonstrated, the key triad genes for 3-GP and 4-GB utilization (i.e. transport, catabolism and gene regulation) appear to have been derived from a common set of origins via gene duplication, and subsequent co-evolution to co-ordinately develop specificity to the relevant compounds. The absence of a 3-GP catabolic system in other closely related *Pseudomonas* species (Yorifuji *et al.*, 1983), and the highest similarity between GpuA and GbuA among the orthologous group of the arginase/agmatinase proteins (<http://www.ncbi.nih.gov/COG>), imply that such genetic events occurred after the divergence of *P. aeruginosa* species, thus establishing independent and efficient catabolic 3-GP and 4-GB systems in this species. Alternatively, *Pseudomonas* species other than *P. aeruginosa* have lost the 3-GP utilization system during or after divergence from an ancestor.

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