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

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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/amaгинase 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 *gbuA*. GbuA catalyses the specific hydrolysis of 3-GP into \(\beta\)-alanine and urea (Yorifuji & Sugai, 1978); the former product can be...
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-GBP 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 gbu-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-GBP, we cloned the gbu-9018 locus, and identified three genes (gpuPAR) that are required for 3-GBP utilization. Structural and functional analyses of these genes established that gbuA is an allele of gbu-9018, that gpuP encodes a 3-GBP transport protein having homology to PA1418 (a gene downstream of gbuA), and that gpuR specifies a transcription activator of gpuA. The striking sequence similarity between the Gpu and Gbu counterparts implies that the gbu and gbuA gene trios have evolved from common triad ancestors.

**METHODS**

**Strains, plasmids and media.** *P. aeruginosa* PAO1 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* PAO1 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 gbu-9018 locus.** A plasmid library of Sau3AI 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 plasmid libraries 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-GBP-utilizing (Gpu$^+$) transconjugants on MPP agar containing 125 μg carbenicillin ml$^{-1}$, and 20 mM 3-GBP 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 gbu-9018 fragment.

**Plasmid and strain construction.** To localize gbu-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-kb BglII–HindIII 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 PromH site of PA288 as a Smal fragment, and into the BamH1 site of PA287, as a BamH1 fragment, created plasmids pYI1041 and pYI1043, respectively (Fig. 2b). Similarly, insertion of an ßSp/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 BglII 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 BamH1, PvuII and Smal 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$^+$ transconjugants harbouring the plasmid sequences integrated at the corresponding chromosome locus by recombination were selected on LB agar containing gentamicin (100 μg ml$^{-1}$). Subsequent selection on LB agar 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 = gbuA::Gm) and PAO4524 (PA287 = gpuP::Gm). Correct insertion of the Gm cassette and ßSp/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 (OD$_{600}$ = 0.3) in MMP containing the indicated carbon and nitrogen sources at 20 mM, as described (Nishijyo et al., 2001). Samples of RNA (50 μg) 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

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<th>Strain or plasmid</th>
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| **Strains**
| *P. aeruginosa* |                  |                     |
| PAO1              | Wild-type        | Stover *et al.* (2000) |
| PAO4173           | *gpuA*9005 *gpu*1018 | Nakada & Itoh (2002) |
| PAO4520           | *gpuR*:Gm        | This study          |
| PAO4522           | *gpuA*::Gm       | This study          |
| PAO4524           | *gpuP*::Gm       | This study          |
| **E. coli**       |                  |                     |
| HB101             | *supE44* hisS20(rl− mK+) recA13 ara-14 proA2 lacY galK2 rpsL20 xyl-5 mtl-1 | Sambrook *et al.* (1989) |
| XL-1 Blue         | endA1 gyrA96 thi-1 hsdR17 *supE44* recA1 lac[F′, proA8 lacP/ZM15 Tn10 (TcR)] | Stratagene          |
| **Plasmids**      |                  |                     |
| pRK2013           | KmR, ColE1 replicon, tra (RK2) | Comai *et al.* (1983) |
| pPS858            | ApR, GmR, ColE1 replicon carrying a Gm cassette | Hoang *et al.* (1998) |

(Amersham Pharmacia Biotech) using a GenVac Blotter (Pharmacia LKB). Transcripts of *gpuPAR* 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 *EcoO1091*–*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 μg) were annealed with oligonucleotides 5′-GGCGATGAAAGTGGGGAATCCCGGGAAGG-3′, complementary to the region at nt +48 to +75 of *gpuA*; and 5′-CGAGGAAAATCGCTCTGGCTCTTCGCCTTG-3′, complementary to the region at nt +72 to +100 of *gpuP*, and end-labelled with [α-32P]dATP (220 TBq mmol⁻¹). (Amersham Pharmacia Biotech) using polyribo nucleotide kinase (Takara Bio). A complementary strand was synthesized using avian reverse transcriptase (RAV-2; Takara Bio) in the presence of deoxyri bonucleotides. 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 pYJ104 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/P* 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/P* 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/P* 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% agarose gels by electrophoresis, and stained with ethidium bromide.

**Enzyme assays.** We prepared extracts from cells growing exponen tially (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 μmol 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 *gpuPAR* genes**

We cloned a 4.5 kb *SalI* fragment carrying the *gpu-9018* locus into plasmid pYJ104 (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*, 48 461...
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, Mr 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, Mr 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 Mr 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

![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, BamHI; Bg, BglII; E, EcoRI; H, HindIII; Pv, PvuII; Sm, SmaI; Su, Sau3A1 (only sites used in construction of pYJ104 are indicated); X, XhoI.](image-url)
pYI104, 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 (Mr = 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 BamHI site of gpuP, and into the SalI 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::ΩSp/Sm), pYII1057 (gpuP::ΩSp/Sm) and pYII1060 (PA286::ΩSp/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_gpuR exist, and suggest that some transcription from P_gpuR 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.](http://mic.sgmjournals.org/4059)
Identification of the P<sub>gpuA</sub> and P<sub>gpuP</sub> promoters

To localize the <i>gpuA</i>, <i>gpuP</i> and <i>gpuR</i> 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 <i>gpuA</i> transcript, and one for the <i>gpuP</i> transcript (Fig. 5a, and b). However, a cDNA for the <i>gpuR</i> 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 <i>gpuA</i> transcripts at 296 and 299 bp upstream from the translation initiation ATG codon of <i>gpuA</i> (Fig. 5a), allowing identification of the −35 (5'-TGGTCA-3') and −10 (5'-GAGCAT-3') sequences of P<sub>gpuA</sub> at around 1080 nt of the <i>gpuA</i> coding region (1386 nt). The 5' end of the <i>gpuR</i> transcript was located 53 bp upstream of the translation initiation ATG codon of this gene (Fig. 5b), positioning the −35 (5'-TTCAAG-3') and −10 (5'-TATCTT-3') sequences of P<sub>gpuR</sub> at appropriate distances from the 5' end. The −10 and −35 sequences of these promoters resembled those for the σ<sup>70</sup> RNA polymerase holoenzyme.

**DISCUSSION**

Peptide mapping has revealed some similarity between the GpuA and GbuA sequences (Yorifuji <i>et al</i>, 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 <i>E. coli</i> argatinase (the speB product) involved in putrescine synthesis (Glasdorff, 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 <i>P. aeruginosa</i> PAO1 indicates that this strain has no speB. In fact, this strain recruits agmatine deaminase (the aguA product) and N-carbamoylputrescine amidohydrolase (the aguB product), which were discovered as the catabolic enzymes of agmatine (Nakada <i>et al</i>, 2001) in the biosynthetic conversion of agmatine to putrescine (Nakada & Itoh, 2003). A detailed phylogenetic analysis (Sekowska <i>et al</i>, 2000) has divided the arginase/agmatinase family of proteins into arginasins, agmatinases, and a third group of plant and bacterial enzymes, including the SpeB1 and SpeB2 of <i>P. aeruginosa</i> PAO1. Our previous identification of SpeB2 as GbuA assumed that the third group consists of amidohydrolases that act upon 4-GB or other guanidino acids (Nakada & Itoh, 2002). The present identification of SpeB1 as GpuA supports this assumption.

The <i>PA1418</i> product has apparent similarity (58 % identity) to GpuP, and this gene is located near <i>gbuAR</i> (Nakada & Itoh, 2002), implying its involvement in 4-GB uptake. However, <i>PA1418</i> knockout minimally affects the Gbu<sup>+</sup> phenotype (Nakada & Itoh, 2002). Thus, <i>P. aeruginosa</i> PAO1 must have a 4-GB transport gene that is not linked to <i>gbuAR</i>. If <i>PA1418</i> encodes a 4-GB transport protein, a second transport gene would hinder the effect of the <i>PA1418</i> mutation on 4-GB utilization. Therefore, a possible role of <i>PA1418</i> in 4-GB transport cannot be ruled out at this stage. To clarify the role of <i>PA1418</i> in 4-GB import, the second transport gene should be identified.
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-acid identity (37 %) of the proteins. The 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-acid identity (37 %) of the proteins.

Fig. 5. Primer extension analysis of gpuA (a) and gpuP (b) transcripts. Complementary strands were synthesized using 32P-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.

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 [AG −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 GbuR 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 coordinately 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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