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Direct Glutaminyl-tRNA Biosynthesis and Indirect Asparaginyl-tRNA Biosynthesis in *Pseudomonas aeruginosa* PAO1

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The genomic sequence of *Pseudomonas aeruginosa* PAO1 was searched for the presence of open reading frames (ORFs) encoding enzymes potentially involved in the formation of Gln-tRNA and of Asn-tRNA. We found ORFs similar to known glutamyl-tRNA synthetases (GluRS), glutaminyl-tRNA synthetases (GlnRS), aspartyl-tRNA synthetases (AspRS), and trimeric tRNA-dependent amidotransferases (AdT) but none similar to known asparaginyl-tRNA synthetases (AsnRS). The absence of AsnRS was confirmed by biochemical tests with crude and fractionated extracts of *P. aeruginosa* PAO1, with the homologous tRNA as the substrate. The characterization of GluRS, AspRS, and AdT overproduced from their cloned genes in *P. aeruginosa* and purified to homogeneity revealed that GluRS is discriminating in the sense that it does not glutamylate $tRNA^{Gln}$, that AspRS is nondiscriminating, and that its Asp-$tRNA^{Asn}$ product is transamidated by AdT. On the other hand, $tRNA^{Gln}$ is directly glutaminylated by GlnRS. These results show that *P. aeruginosa* PAO1 is the first organism known to synthesize Asn-tRNA via the indirect pathway and to synthesize Gln-tRNA via the direct pathway. The essential role of AdT in the formation of Asn-tRNA in *P. aeruginosa* and the absence of a similar activity in the cytoplasm of eukaryotic cells identifies AdT as a potential target for antibiotics to be designed against this human pathogen. Such novel antibiotics could be active against other multidrug-resistant gram-negative pathogens such as *Burkholderia* and *Neisseria* as well as all pathogenic gram-positive bacteria.

The formation of correctly aminoacylated tRNAs is the central step of the faithful translation of the genetic code. Some organisms use at least a distinct aminoacyl-tRNA synthetase (aaRS) for each amino acid species to be charged on the cognate tRNA(s). This is the case in the cytoplasm of eukaryotic cells and in some eubacteria such as *Escherichia coli*, which in addition to its basic set of 20 aaRSs, has an additional lysyl-tRNA synthetase (12). On the other hand, many organisms lack one or several aaRSs (35) and correctly aminoacylate the corresponding tRNA(s) via multistep pathways, such as the transamidation pathway for Gln-$tRNA^{Gln}$ formation present in all archaea and in most bacteria (37, 38); the first step of this pathway is the misacylation of $tRNA^{Gln}$ with glutamate, catalyzed by a nondiscriminating glutamyl-tRNA synthetase (GluRS-ND) (20), followed by the transamidation of Glu-$tRNA^{Gln}$ into Gln-$tRNA^{Gln}$, catalyzed by a tRNA-dependent amidotransferase (AdT) (5). The presence of such alternate pathways for the correct aminoacylation of certain tRNAs reflects the formation of the extant 20 aaRSs by the divergent evolution of the ancestors of the two unlinked classes of aaRSs; for instance, glutaminyl-tRNA synthetase (GlnRS) evolved from a GluRS-ND in primitive eukaryotes that used the transamidation pathway for Gln-$tRNA^{Gln}$ formation (18, 30). There may be physiological reasons for the conservation of such ancestral pathways by some organisms. In some cases, pathways may be redundant; when AdT and asparaginyl-tRNA synthetase (AsnRS) are present (as in gram-positive bacteria), aspartyl-tRNA synthetases (AspRS) could be either discriminating (AspRS-D) or nondiscriminating (AspRS-ND). Similarly, when AdT and GlnRS are present (as in *Pseudomonas aeruginosa*), glutamyl-tRNA synthetase (GluRS) could be either discriminating or nondiscriminating.

In certain archaea (7) and in the gram-negative bacterium *Chlamydia trachomatis* (25), the AsnRS is missing and Asn-$tRNA^{Asn}$ is synthesized by a transamidation pathway involving an AspRS-ND and a heterotrimeric AdT (10). Such AdTs, encoded by the *gatA*, *gatB*, and *gatC* genes, are present in archaea, some bacteria, and most organelles and can transamidate both Glu-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ (3, 5, 6, 25, 27). Heterodimeric AdTs, encoded by the *gatD* and *gatE* genes, are present in some archaea and transamidate only Glu-$tRNA^{Gln}$ (35).

By searching through the complete genomic sequence of the pathogenic bacterium *P. aeruginosa* PAO1 (33) for genes encoding enzymes potentially involved in the formation of Gln-tRNA and of Asn-tRNA, we found those encoding a GluRS, a GlnRS, an AspRS, and an AdT (Fig. 1), but none were similar to the genes encoding known AsnRSs. We report here that *P. aeruginosa* contains a discriminating GluRS, an AspRS-ND, a heterotrimeric AdT, and no AsnRS activity (Fig. 2). It is the first organism where this set of enzymes is shown to be used for the formation of Gln-tRNA and Asn-tRNA. More recently, *gatCAB* has been found to be present and *asnS* was absent in other beta- and lower gamma-proteobacteria such as *Neisseria meningitidis* (24) and *Pseudomonas putida* (23).

MATERIALS AND METHODS

Nucleotide and amino acid sequence analyses. The gapped BLAST algorithm (1) was used for nucleotide sequence analyses of the complete genome of *P. aeruginosa* PAO1 accessible at ftp://www.pseudomonas.com (33). The multiple alignments of amino acid sequences of homologous proteins were made with ClustalX (16) and the Pileup program of Genetics Computer Group, version 10.3

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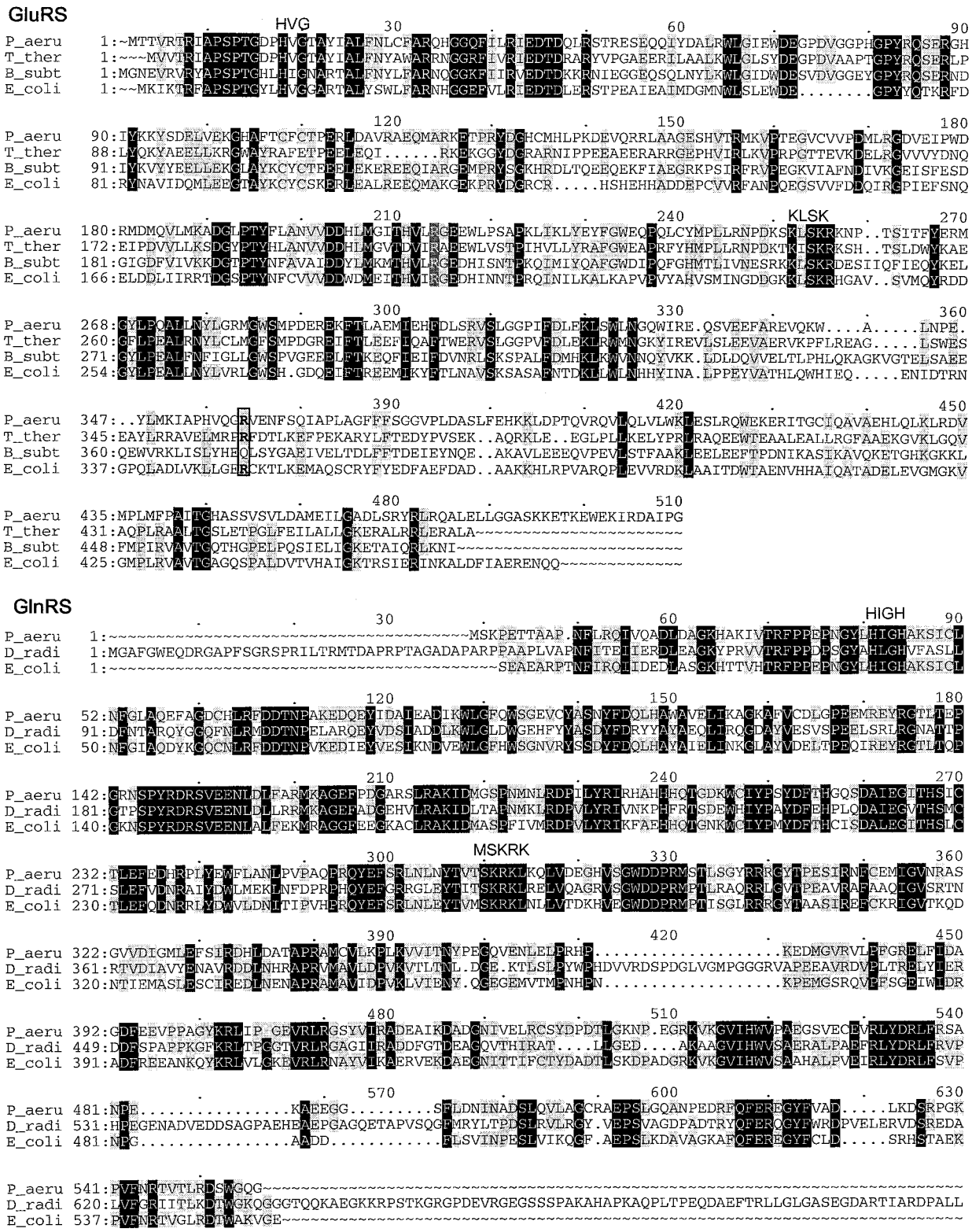

FIG. 1. Multiple-sequence alignments of *P. aeruginosa* PAO1 GluRS, GlnRS, GatC, GatA, GatB, and AspRS with a few respective orthologs from other bacterial species, created by using the Pileup program. The organisms were as follows: P_aeru, *P. aeruginosa* PAO1; T_ther, *T. thermophilus*; B_subt, *B. subtilis*; E_coli, *E. coli*; D_radi, *Deinococcus radiodurans*; C_trac, *C. trachomatis.* The GluRS sequences used were as follows: P_aeru, PA3134; T_ther, P27000; B_subt, P222450; E_coli, P04805. The aligned residues Arg358 of *T. thermophilus* GluRS-D, Arg358 of *P. aeruginosa* PAO1, and GluRS and Gln358 of *B. subtilis* GluRS-ND are boxed. The GlnRS sequences used were as follows: P_aeru, Q9I2U8; D_radi, P56926; E_coli, BAA35328. The GatC, GatA, and GatB sequences used were, respectively, as follows: P_aeru, AAG07870, AAG07871, AAG07872; C_trac, NP_219504, NP_219505, NP_219506; B_subt, O06492, CAB12488, O30509. The AspRS sequences used were as follows: P_aeru, NP_249654; C_trac, O84546; D_radi_D, NP_295070; T_ther_D, P36419; E_coli_D, NP_288303. Residues which were identical in all sequences of each multiple alignment are printed in white on black, and those conserved in at least three of the four GluRS sequences, two of three sequences (GlnRS, GatC, GatA, and GatB), or three or four of the five AspRS sequences are shaded.

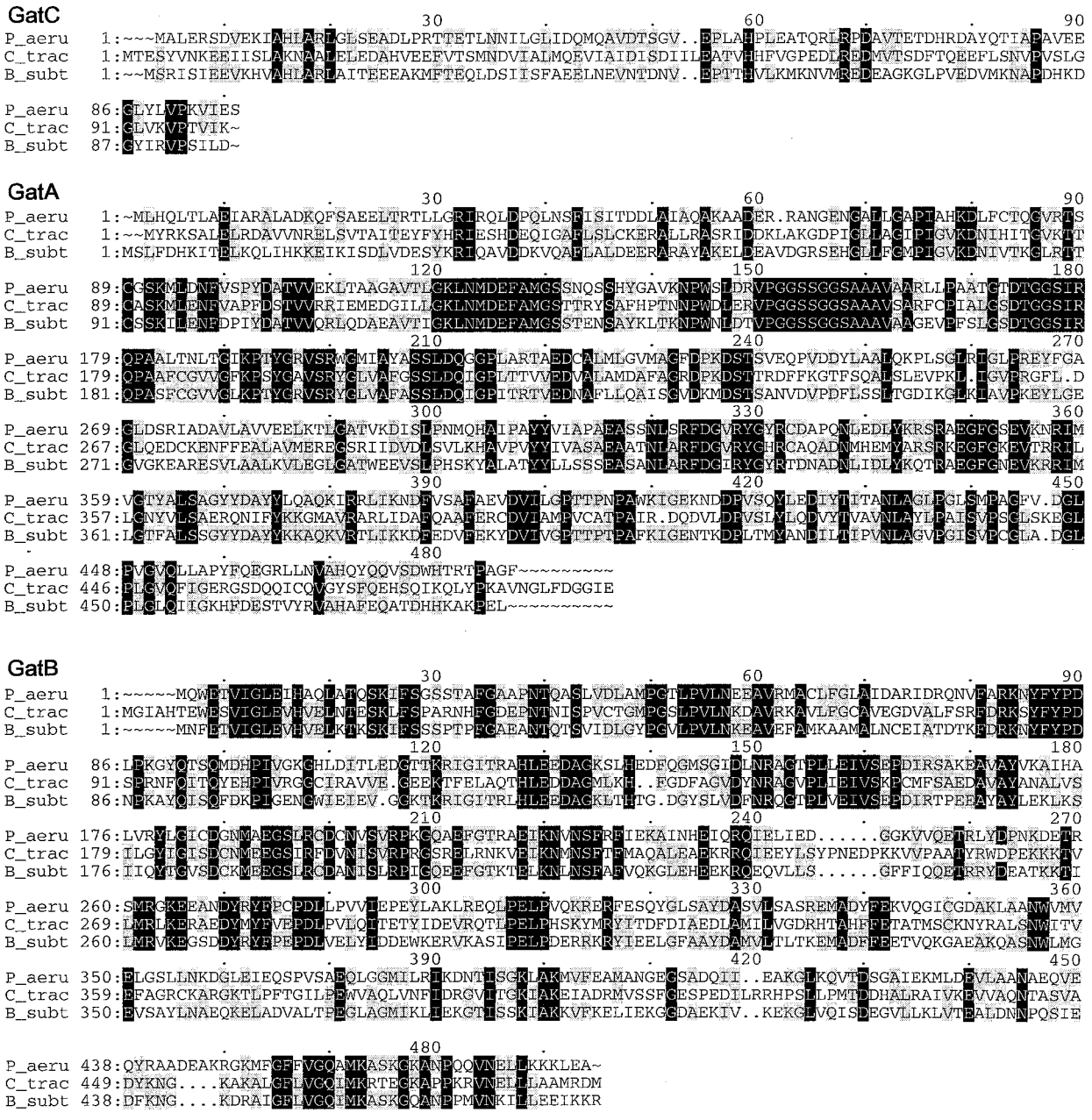

FIG. 1—*Continued*.

(Accelrys Inc., San Diego, Calif.), with the blosum 62 matrix, a gap weight of 10, and a gap length weight of 2.

Bacterial strains and plasmids. *P. aeruginosa* PAO1 (ATCC 15692), kindly provided by Ann Huletsky (Université Laval, Québec, Canada), was used to purify DNA for gene cloning by PCR. *P. aeruginosa* ADD1976, carrying the mini-D180 fragment containing the T7 RNA polymerase gene controlled by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inductible UV-5 promoter (4), was kindly provided by John Mattick (University of Queensland, Brisbane, Australia). The *E. coli-P. aeruginosa* shuttle vectors pUCPSK and pUCPKS were used for protein overproduction. They contain a multiple cloning site, allowing the transcription of the cloned gene from a T7 promoter, and encode a β-lactamase which confers resistance to ampicillin (120 μg/ml) in *E. coli* and to carbenicillin (500 μg/ml) in *P. aeruginosa* (36). The preparation and transformation of competent cells were conducted as described by Irani and Rowe (15).

Cloning of *P. aeruginosa* PAO1 *gltX* and *aspS* genes and of the *gatCAB* operon. The *gltX* gene (1.48 kb) was amplified by PCR from *P. aeruginosa* genomic DNA with the following upstream and downstream oligonucleotides, respectively: 5′-CGCCCCTGAATTCCCGTTTTAACTTCC-3′ and 5′-CCCGGATCCTTATT AGTGGTGGTGGTGGTGGTGGCTGCTGCCGCGGCCCTCGGCGCCGG GAATGGCGTCGCG-3′. It was then inserted into pUCPSK (Fig. 3D). The *aspS* gene (1.77 kb) was similarly amplified by PCR with the following oligonucleotides: 5′-GGCAGCCGGGAGCTCCACAGAA-3′ and 5′-GCTCTTTGCG AAGCTTGATGTTGG-3′. It was then inserted into pUCPKS (Fig. 3E). The *gatCAB* operon was cloned in three steps. First, *gatB* (1.44 kb) was PCR amplified with the oligonucleotides 5′-CCCGGTACCAGGAGGTCTCCATGGGCA GCAGCCACCACCACCACCACCACCACCACAGCAGCGCCGAGGGCC GCATGCAATGGGAAACCG-3′ and 5′-CGAACCCGGC GTCAAGCTTTG ACTCACGCTTC-3′ and cloned into pUCPSK (Fig. 3A). Second, *gatCA* (1.72 kb) was PCR amplified with the oligonucleotides 5′-CGCCCAAGCTTAGGA GGTCTCCATGGGCAGCAGCCACCACCACCACCACCACCACAGC

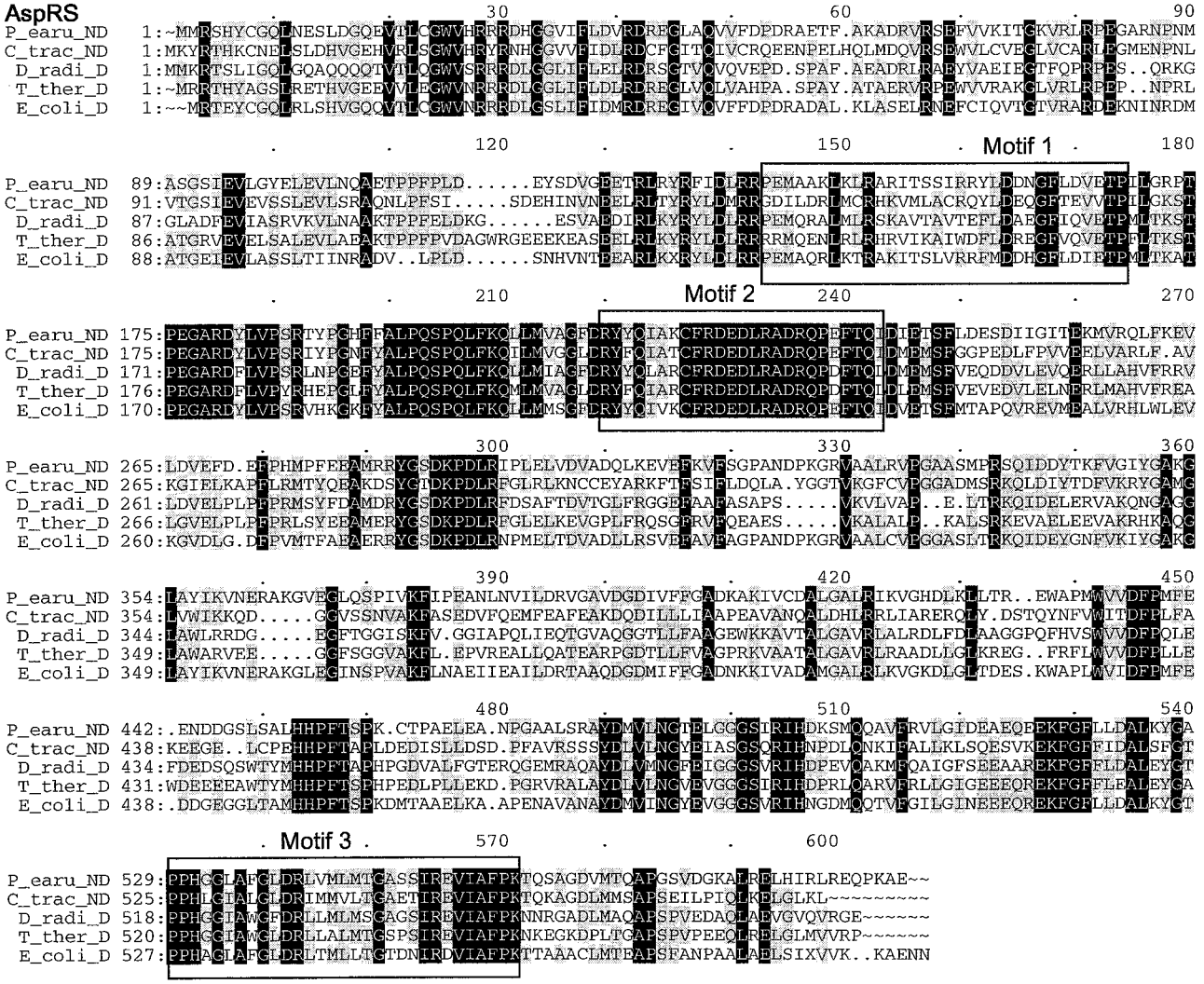

FIG. 1—*Continued*.

AGCGCCGAGGGCCGCATGGCGCTTGAACGCTCCGAC-3′ and 5′-GCTC TAGAGCTTATTAGAAGCCGGCCGGGG-3′ and cloned into pUCPSK (Fig. 3B). Finally, the *Kpn*I-*Hin*dIII fragment of pUCPSK/*gatB* was inserted upstream of *gatC* in pUCPSK/*gatCA*, generating the *gatBCA* operon (3.2 kb) (Fig. 3C). The integrity of the cloned genes was checked by sequencing.

Enzyme purification. *P. aeruginosa* was grown with strong agitation in modified Luria-Bertani medium (2.5 g of NaCl per liter) (C. Hancock, personal communication) to reduce the synthesis of alginate, which interferes with enzyme purification. The overproduced *P. aeruginosa* GluRS and AspRS, carrying C-terminal His tags, were purified to homogeneity by affinity chromatography on nickel nitrilotriacetate (Ni-NTA) superflow agarose (Qiagen). *P. aeruginosa* AdT was overproduced in *P. aeruginosa* ADD1976 carrying the plasmid pUCPSK/*gatBCA*, with His tags on the C and B subunits but none on the A subunit. The AdT purified by Ni-NTA affinity chromatography contains equimolar amounts of each of the three subunits. These purified enzymes were concentrated by filtration on Centricon membranes (YM-5, -10, or -30). *P. aeruginosa* GlnRS was partially purified from a clarified (S-10) crude extract of *P. aeruginosa* PAO1 by partition in a polyethylene glycol-dextran two-phase system, as previously used for *E. coli* GlnRS (17), followed by chromatography on a Q-Sepharose column (Amersham Pharmacia Biotech).

Purification of unfractionated tRNA from *P. aeruginosa* PAO1. Cells were grown as described above to reduce the synthesis of alginate, which strongly interferes with phenol extraction of tRNA. All the operations were conducted at 4°C. The cell pellet was suspended in 20 mM Tris-HCl (pH 7.9) and shaken with an equal volume of phenol previously equilibrated with Tris-HCl (pH 7.9). After separation of the phases by centrifugation, the aqueous phase was submitted to a second phenol extraction. Nucleic acids were precipitated from the resulting aqueous phase by the addition of 0.1 volume of 20% potassium acetate (pH 5.0) and 3 volumes of 95% ethanol. The precipitate was resuspended in 1 M NaCl, which allowed the small RNAs to be solubilized but kept the large ribosomal RNAs as the precipitate. Unfractionated tRNA was then precipitated from the supernatant in the presence of 3 volumes of 95% ethanol at −20°C for at least 30 min, solubilized in sterilized distilled and deionized water, and purified from remaining traces of rRNA by filtration through Centriplus 100,000-molecular-weight membranes (Millipore).

Purification of *P. aeruginosa* tRNA^{Asp}, tRNA^{Asn}, and tRNA^{Gln}. The *P. aeruginosa* genome contains a single species of each of tRNA^{Asp}, tRNA^{Asn}, and tRNA^{Gln}, encoded by four, two, and one genes, respectively (http://rna.wustl.edu/GtRDB/Paer/Paer-summary.html). Each of these tRNA species was purified to homogeneity, as a complex with a complementary oligonucleotide (24-mer), by polyacrylamide gel electrophoresis under nondenaturing conditions and recovered by electroelution (the choice of the complementary oligonucleotides and the detailed experimental conditions will be described elsewhere).

***P. aeruginosa* tRNA aminoacylation.** Unfractionated tRNA, isolated as described above, was aminoacylated with [^{14}C]glutamate, [^{14}C]aspartate, or [^{14}C]glutamine with pure GluRS or AspRS or partially purified GlnRS, respectively. [^{14}C]asparagine was used to search for the presence of an AsnRS. The aminoacylation reactions were conducted at 37°C in 50 mM Na HEPES (pH 7.2), 25 mM $MgCl_2$, 15 mM KCl, 5 mM dithiothreitol, 1 mM ATP, 50 μM unfractionated tRNA, 125 μM amino acid substrate ([^{14}C]glutamate, 238 mCi/mmol;

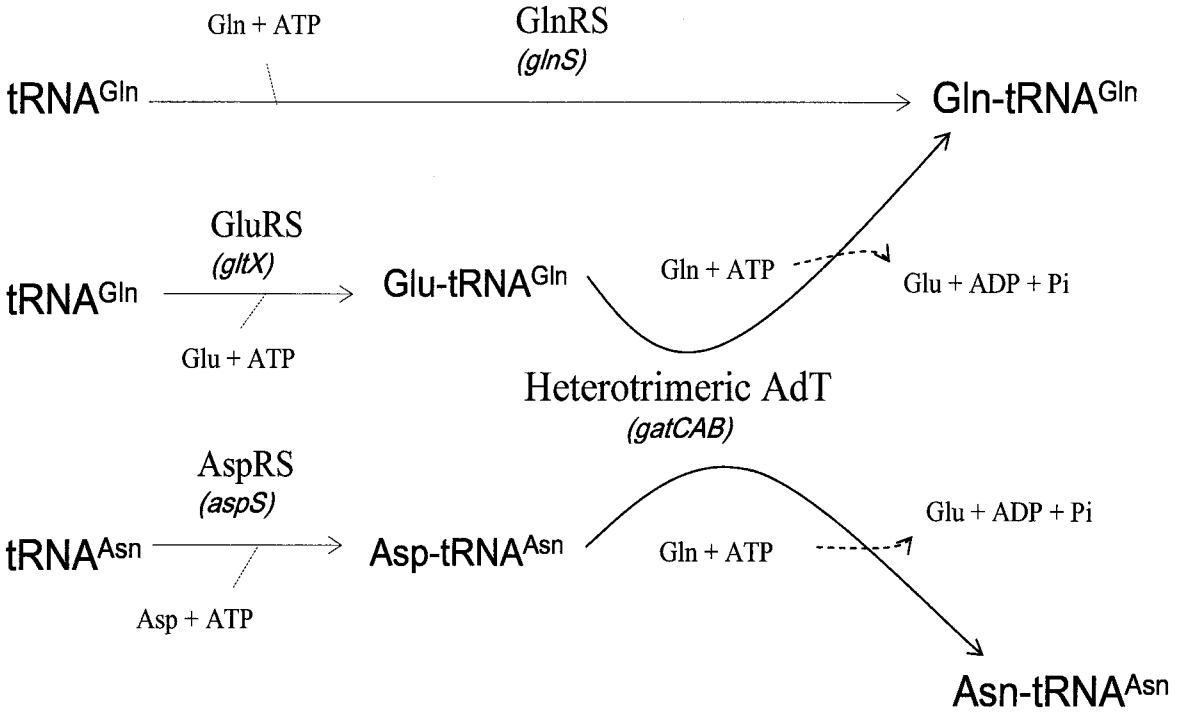

FIG. 2. Putative pathways for Gln-tRNA^{Gln} and Asn-tRNA^{Asn} synthesis in *P. aeruginosa* PAO1 based on the genes identified in its complete genomic sequence and without excluding the possibility that an atypical AsnRS is present.

[^{14}C]glutamine, 244 mCi/mmol; [^{14}C]aspartate, 216 mCi/mmol; or [^{14}C]asparagine, 210 mCi/mmol), and the enzyme (4.2 μg of pure GluRS or AspRS/ml or 12.5 μg of partially purified GlnRS/ml). The ^{14}C-labeled amino acids were purchased from Amersham Pharmacia Biotech.

Asp-tRNA^{Asn} and Glu-tRNA^{Gln} transamidation reactions. The transamidation reactions were conducted at 37°C in 50 mM Na HEPES (pH 7.2), 25 mM $MgCl_2$, 15 mM KCl, 5 mM dithiothreitol, 1 mM ATP, 50 μM unfractionated tRNA, and 125 μM [^{14}C]glutamate or [^{14}C]aspartate, as described by Curnow et al. (5) and Raczniak et al. (25). In a preliminary step, the aminoacyl-tRNA substrates for AdT were prepared by adding either AspRS or GluRS to this mixture and incubating it for 15 min at 37°C, which led to plateaux of Asp-tRNA and Glu-tRNA. The transamidation reaction was then started by the addition of 320 μg of pure AdT/ml and 5 mM unlabeled glutamine. The reaction was carried out for 30 min at 37°C and was stopped by the addition of 2.5 M Na acetate (pH 5.2) to a final concentration of 0.3 M. This reaction mixture was shaken with 1 volume of phenol at pH 5.2 and 1 volume of chloroform. After centrifugation, tRNA was ethanol precipitated from the aqueous phase, washed with 70% ethanol, dried and solubilized in 50 μl of 25 mM KOH, and incubated for 1 h at 65°C to deacylate aminoacyl-tRNAs. This solution was then neutralized by the addition of 1.3 μl of 100 mM HCl and dried under vacuum. The residue was solubilized in 10 ml of distilled and deionized water, and 2 μl of this solution was analyzed for its amino acid content by ascending chromatography on microcrystalline cellulose thin-layer plates (Whatman) with ammonia-water-chloroform-methanol (2:1:6:6) as described by Curnow et al. (5). The thin-layer chromatography (TLC) plate was then dried at room temperature, and the position and radioactivity of the spots were measured and analyzed with a Fuji BAS 1000 phosphorimager, with the Image gauge, version 4.0 software.

RESULTS

***P. aeruginosa* genes encoding enzymes potentially involved in the formation of Gln-tRNA and Asn-tRNA.** Analysis of the complete genomic sequence of *P. aeruginosa* PAO1 (33) (ftp://www.pseudomonas.com) by gapped BLAST (1), ClustalX (16), and version 10.3 of the Genetics Computer Group (Accelrys Inc.) revealed the presence of open reading frames (ORFs) similar to known GluRSs, GlnRSs, AspRSs, and trimeric AdT (Fig. 1) but none similar to known AsnRSs. As trimeric AdT characterized in other bacteria can transamidate both Glu-tRNA^{Gln} and Asp-tRNA^{Asn} (3, 5, 25), this set of genes suggests the existence of two pathways of Gln-tRNA^{Gln} formation (direct, via GlnRS; indirect, via GluRS-ND and AdT) and of only the indirect pathway for Asn-tRNA^{Asn} formation. However, firm conclusions about the pathways used in *P. aeruginosa* for the formation of Glu-tRNA^{Gln} and Asp-tRNA^{Asn} cannot rely only on the presence of these genes, first because it is not possible at this point to determine from its amino acid sequence whether a GluRS is discriminating or nondiscriminating and second because the absence of a gene similar to the known *asnS* genes does not imply that *P. aeruginosa* has no AsnRS activity. Indeed, some aaRSs such as class I Lys-tRNA synthetase of *Methanococcus maripaludis* (13) and Cys-tRNA synthetase of *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum* (9, 32) are too different from their cognate aaRSs in other organisms to be identified by sequence comparisons. The same set of genes encoding enzymes potentially involved in the formation of Gln-tRNA and Asn-tRNA (Fig. 2) is also present in all beta- and some gamma-proteobacteria whose complete genomes have been reported: *P. aeruginosa* (gamma), *P. putida* (gamma), *Pseudomonas syringae* (gamma), *Neisseria gonorrhoeae* (beta), *N. meningitidis* (beta), *Nitrosomonas europaea* (beta), *Bordetella pertussis* (beta), *Ralstonia solanacearum* (beta), and *Burkholderia pseudomallei* (beta). No biochemical characterization of these enzymes has been reported. Therefore, we undertook the search for AsnRS activity in *P. aeruginosa* and the biochemical characterization of its AspRS, GluRS, GlnRS, and AdT.

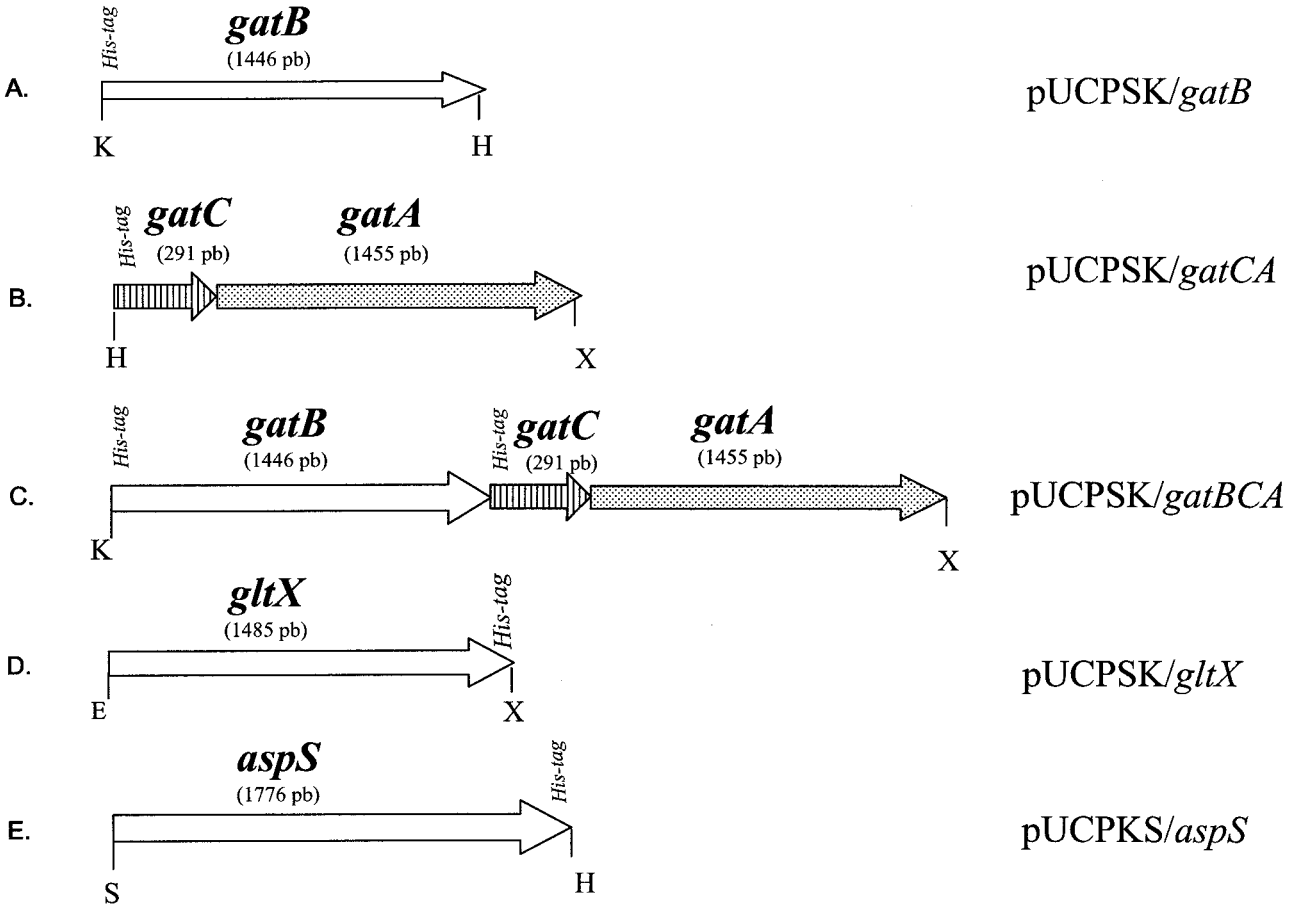

FIG. 3. *P. aeruginosa* PAO1 genes amplified by PCR and inserted into the multiple cloning site of the *E. coli-P. aeruginosa* shuttle vector pUCPSK or pUCPKS, which differ only by the orientation of their multiple cloning sites (36). The resulting vectors, identified on the right, express the inserted gene(s) from a proximal T7 promoter. The letters K, H, X, E, and S represent the *Kpn*I, *Hin*dIII, *Xba*I, *Eco*RI, and *Sac*I restriction sites, respectively. Upstream of *gatB* and *gatC*, we inserted by PCR the Shine-Dalgarno sequence AGGAGG frequently found in *P. aeruginosa*, 8 His codons (CAC), and a sequence encoding the factor Xa digestion site Arg-Glu-Gly-Arg (with codons preferentially used in *P. aeruginosa*). Downstream of *gltX* and *aspS*, we inserted a sequence encoding the factor Xa digestion site, 6 His codons, and two stop codons.

Absence of an AsnRS in *P. aeruginosa*. To detect AsnRS activity in crude extracts of *P. aeruginosa*, we measured the incorporation of [^{14}C]asparagine into homologous unfractionated tRNA and observed the incorporation of the ^{14}C label into this tRNA fraction, even in the presence of unlabeled aspartate; however, identification of the charged amino acid by TLC, following the deacylation of the charged tRNAs, revealed that it was [^{14}C]aspartate. This result does not rule out the presence of AsnRS activity, since very active asparaginases in the crude extract could rapidly transform [^{14}C]asparagine into [^{14}C]aspartate (31). We thus fractionated *P. aeruginosa* PAO1 extracts by several chromatographic steps, selecting the fractions endowed with the above-mentioned activity. We found that the purest active fraction contained AspRS and the GatA and GatB subunits of the heterotrimeric AdT. In the presence of this fraction and of [^{14}C]asparagine, only [^{14}C]aspartate was found acylated to tRNA, indicating that aspartate was formed by an asparaginase activity, and charged on tRNA by the copurified AspRS. No AsnRS activity was detected in pure AspRS nor in pure AdT. Therefore, we conclude that *P. aeruginosa* does not contain an AsnRS.

Cloning and (over)expression of the *P. aeruginosa* genes encoding GluRS, AspRS, and AdT in *E. coli* and *P. aeruginosa*. The *P. aeruginosa* PAO1 *gltX* and *aspS* genes, flanked at their 3′ ends by His tag-encoding extensions, have been amplified by PCR from genomic DNA and cloned into the *P. aeruginosa-E. coli* multicopy (about 14 copies per cell) shuttle vector pUCPSK (36) under the control of a T7 promoter (Fig. 3A and B). The *E. coli* thermosensitive strain JP1449 altered in *gltX* (19, 26), which does not contain the T7 RNA polymerase gene, grows at restrictive temperature (42°C) when transformed with pUCPSK/*gltX* but does not grow under these conditions when transformed with pUCPKS alone. This result shows that even in the absence of T7 RNA polymerase, the level of expression of *P. aeruginosa gltX* provides enough GluRS to complement the temperature-sensitive character of this *E. coli* mutant and suggests that *P. aeruginosa* GluRS is discriminating. On the other hand, no transformants of *E. coli* BL21(DE3) (which carries an IPTG-inducible T7 RNA polymerase gene) with pUCPSK/*gltX* were obtained, indicating the toxicity for *E. coli* of high levels of *P. aeruginosa* GluRS. The mechanism responsible for this toxicity is yet unknown. On the other hand, no

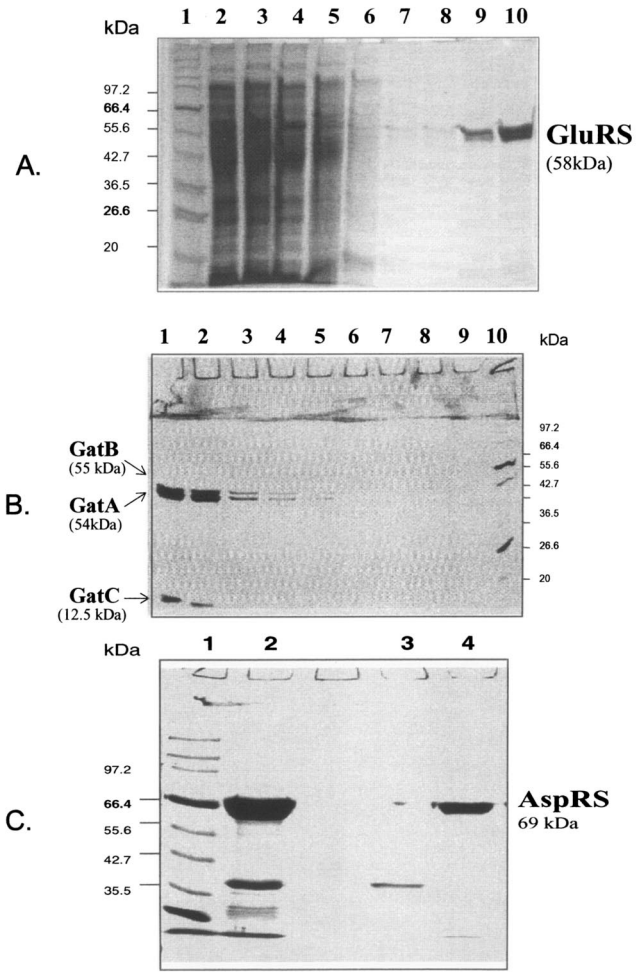

FIG. 4. SDS-PAGE characterization of *P. aeruginosa* PAO1 GluRS (A), the three subunits of the heterotrimeric AdT (B), and AspRS (C) overproduced in *P. aeruginosa* ADD1976 and purified by affinity chromatography on Ni-NTA. (A) Lanes: 1, protein standard; 2 to 8, wash with 30 mM imidazole; 9 to 10, GluRS elution with 90 mM imidazole. (B) Lanes: 1 to 9, amidotransferase elution with 100 mM imidazole, after an initial wash with 30 mM imidazole; 10, protein standard. (C) Lanes: 1, protein standard; 2, AspRS elution with 85 mM imidazole, after an initial wash with 30 mM imidazole; 3 and 4, contaminant and pure AspRS, respectively, removed after Superdex 200 chromatography. Numbers indicate the molecular mass (in kilodaltons) of protein standards. The gels (8% polyacrylamide) are stained with Coomassie blue.

transformants by the pUCPSK/*aspS* of *E. coli* DH5α or CS89 (29), which carries a thermosensitive AspRS, were obtained, suggesting that *P. aeruginosa* AspRS is nondiscriminating. Therefore, we overproduced *P. aeruginosa* GluRS and AspRS in *P. aeruginosa* ADD1976 and purified these His-tagged enzymes to near homogeneity by affinity chromatography (Fig. 4A and C). As removing His tags from the purified GluRS or AspRS did not significantly affect the activity, we used the His-tagged forms in the work described below.

We did not succeed in cloning *gatCAB* in the same vector after its amplification by PCR, probably because of the large size of this operon (3.5 kbp). Independent clonings of *gatCA* and *gatB* were successful (Fig. 3A and B) and allowed the overproduction in *P. aeruginosa* ADD1976 of the His-tagged B subunit of AdT and its purification by affinity chromatography. However, the untagged A subunit, overproduced in the same cells as the His-tagged C subunit, was not retained during the affinity chromatography. This indicates that the interaction between A and C is weak, at least in the absence of B. Therefore, we inserted *gatB* upstream of *gatC* in the pUCPSK/*gatCA* (see Materials and Methods) to overproduce the three subunits in the same cell (Fig. 3B). Although the expression of this artificial *gatBCA* operon in *E. coli*(DE3) did not affect the growth of this host, we conducted *gatBCA* overexpression in *P. aeruginosa* ADD1976; purification of the overproduced *P. aeruginosa* AdT by affinity chromatography yielded equimolar ratios of the three subunits (Fig. 4B).

***P. aeruginosa* has GlnRS activity, and its GluRS is discriminating.** The presence of GlnRS activity was detected in the supernatant of a crude extract (centrifugation at 10,000 × *g*). This GlnRS was partially purified by Q-Sepharose chromatography and used to charge unfractionated tRNA from *P. aeruginosa* with [^{14}C]glutamine (Fig. 5A). From the value of the plateau obtained, we calculated that 2.4% of these tRNA molecules accept glutamine. Moreover, we verified by chromatography that glutamine, and not glutamate, was acylated to tRNA (Fig. 5D). This result excludes the possibility that $tRNA^{Gln}$ was glutaminylated via the transamidation pathway involving a GluRS-ND and AdT, because in that case both glutamate and glutamine would have been found to be acylated to unfractionated tRNA. This control was made to exclude the possibility that the ^{14}C-labeled amino acid measured in this experiment is glutamic acid. This could have happened by the conversion of [^{14}C]glutamine into [^{14}C]glutamate by a glutaminase in the partially purified GlnRS fraction followed by the charging of [^{14}C]glutamate on tRNA. The presence in the *P. aeruginosa* genome of an ORF similar to known *glnS* genes is not sufficient to conclude that this ORF encodes a GlnRS because GluRSs and GlnRSs are closely related (11, 39), and some bacteria have no GlnRS and two GluRSs (one is discriminating and the other is nondiscriminating) (34). The presence of only glutamine acylated to tRNA (Fig. 5D) in that experiment demonstrates the existence of a GlnRS activity in *P. aeruginosa.* Finally, $tRNA^{Gln}$ purified to homogeneity by gel electrophoresis (see Materials and Methods) is glutaminylated by this GlnRS fraction (Fig. 5B).

The pure GluRS charges 6.4% of unfractionated tRNA from *P. aeruginosa* with [^{14}C]glutamate (Fig. 5C). When this [^{14}C]glutamyl-tRNA was incubated in the presence of pure AdT from *P. aeruginosa*, no [^{14}C]glutaminyl-tRNA was formed (Fig. 6A). Moreover, *P. aeruginosa* GluRS does not aminoacylate pure $tRNA^{Gln}$ from *P. aeruginosa* (results not shown). These results demonstrate that *P. aeruginosa* GluRS is discriminating.

***P. aeruginosa* AspRS is nondiscriminating.** The pure AspRS charges 6.9% of unfractionated tRNA from *P. aeruginosa* with [^{14}C]aspartate (Fig. 5E). When this [^{14}C]aspartyl-tRNA was incubated in the presence of pure AdT from *P. aeruginosa*, about 34% was transformed into [^{14}C]asparaginyl-tRNA (Fig. 6C). This result demonstrates that *P. aeruginosa* AspRS is nondiscriminating and indicates that the 6.9% of the tRNA molecules charged with aspartate correspond to about 4.6% $tRNA^{Asp}$ and 2.3% $tRNA^{Asn}$. These proportions are consis-

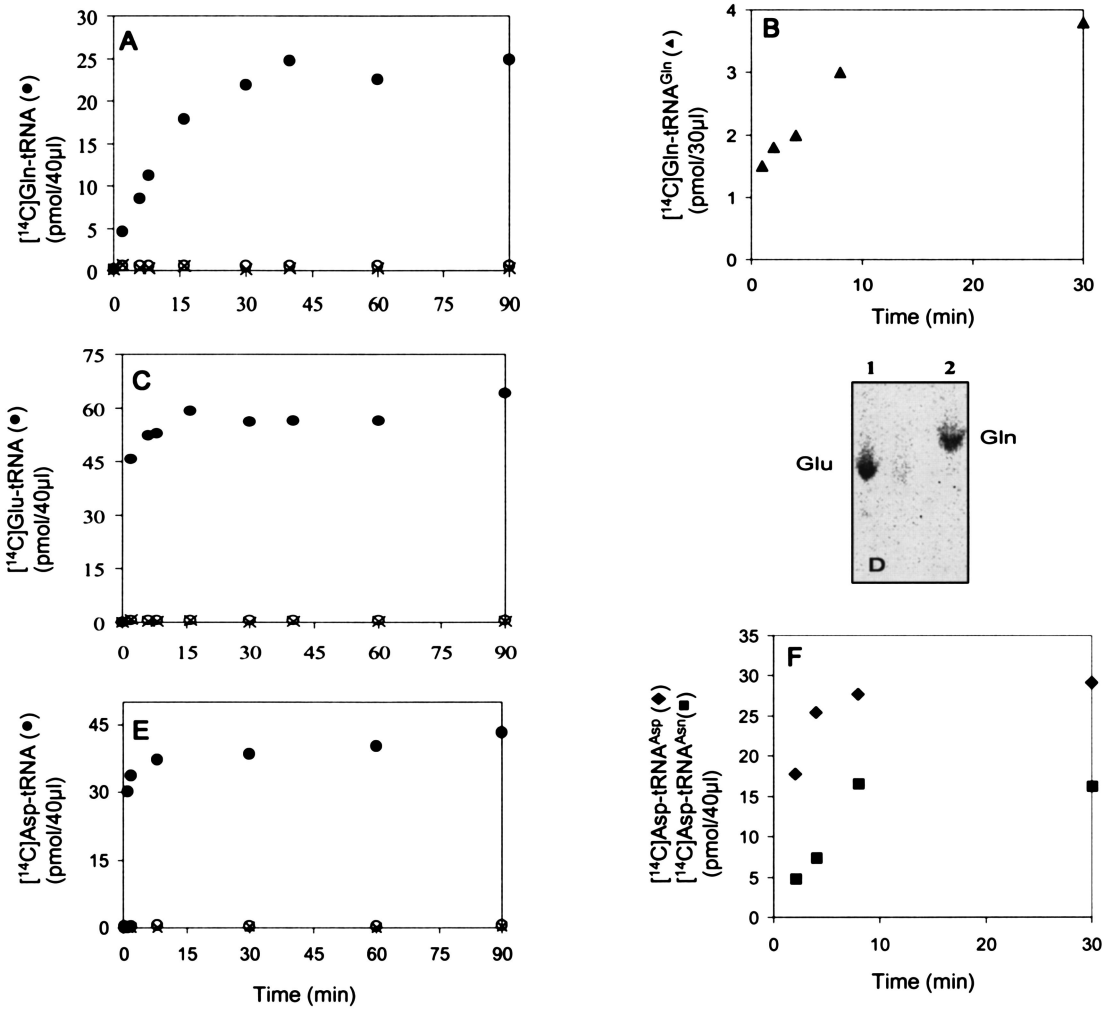

FIG. 5. Aminoacylation of unfractionated tRNA, pure $tRNA^{Gln}$, $tRNA^{Asp}$, and $tRNA^{Asn}$ from *P. aeruginosa* PAO1. The reactions shown in panels A, C, and E were conducted in the presence of unfractionated tRNA and catalyzed by partially purified GlnRS, pure GluRS, and pure AspRS from *P. aeruginosa* PAO1, respectively. Panel B shows the glutaminylation of pure $tRNA^{Gln}$ by partially purified GlnRS, and panel F shows the aspartylation of pure $tRNA^{Asp}$ and $tRNA^{Asn}$ by pure AspRS. (D) Identification by TLC (see Methods) of the amino acid charged on tRNA by GluRS in the reaction shown in panel C (lane 1) and by GlnRS in the reaction shown in panel A (lane 2). Shown are complete reactions (filled circles) and controls without either tRNA (X) or enzyme (empty circles).

tent with the presence of 4 $tRNA^{Asp}$ genes and 2 $tRNA^{Asn}$ genes in the genome of this bacterium (http://rna.wustl.edu/GtRDB/Paer/Paer-summary.html) and with the observation that the levels of individual tRNA genes in bacteria are generally controlled by gene dosage (8, 14). Finally, the nondiscriminating character of *P. aeruginosa* AspRS is demonstrated directly by its capacity to aspartylate efficiently both pure $tRNA^{Asp}$ and $tRNA^{Asn}$ from *P. aeruginosa* (Fig. 5F).

The heterotrimeric AdT of *P. aeruginosa* has no Glu-$tRNA^{Gln}$ substrate in its host, but it can transamidate heterologous Glu-$tRNA^{Gln}$. The pure heterotrimeric AdT of *P. aeruginosa* transamidates its homologous Asp-$tRNA^{Asn}$ into Asn-$tRNA^{Asn}$ (Fig. 6C). The characterized heterotrimeric AdTs can use both Asp-$tRNA^{Asn}$ and Glu-$tRNA^{Gln}$, even if the latter substrate is lacking in *P. aeruginosa* because of the discriminating character of its GluRS (Fig. 6A), its AdT has kept its capacity to transamidate Glu-$tRNA^{Gln}$, as shown with the corresponding tRNA of *Bacillus subtilis* (Fig. 6B).

DISCUSSION

By analyzing the genome of *P. aeruginosa* and characterizing several enzymatic activities potentially involved in the formation of Gln-tRNA and of Asn-tRNA, we have found that it does not have an AsnRS and that it is the first known organism to synthesize Gln-tRNA, via the direct pathway (GlnRS), and Asn-tRNA, via the transamidation pathway. Up to now, the absence of AsnRS was correlated to the absence of GlnRS in archaea (7) and in *Chlamydia* (25). Genomic data indicate that all beta- and some gamma-proteobacteria have a GlnRS and lack an AsnRS.

The presence of a GlnRS in *P. aeruginosa* is consistent with

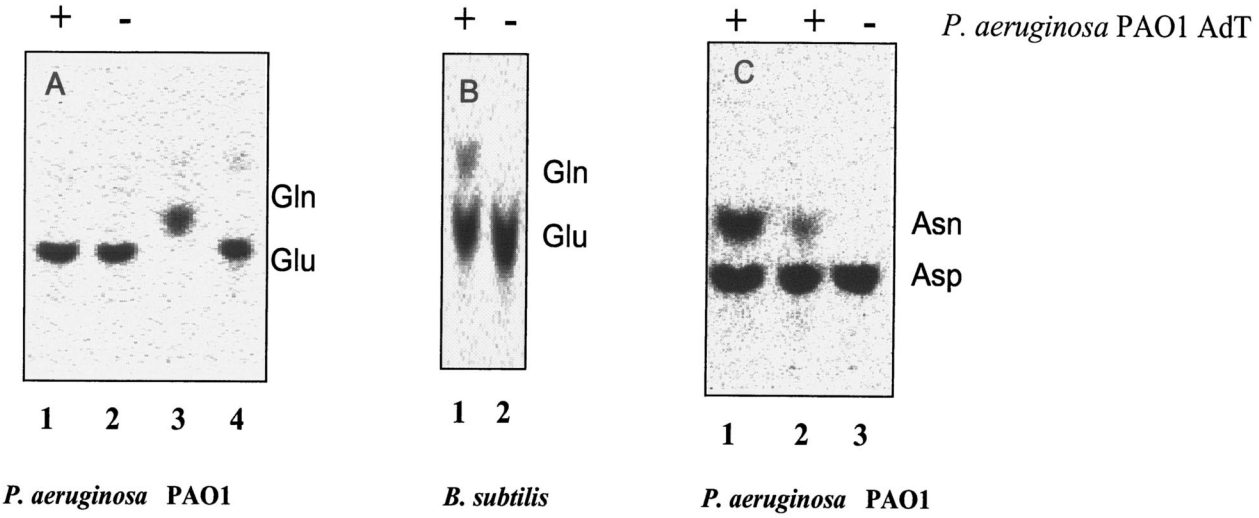

FIG. 6. Activity of *P. aeruginosa* PAO1 heterotrimeric AdT. (A) *P. aeruginosa* Glu-tRNA. Lanes: 1, with (+) AdT; 2, without (−) AdT; 3 and 4, glutamine and glutamate, respectively, as standards. (B) *B. subtilis* Glu-tRNA. Lanes: 1, with AdT; 2, without AdT. (C) *P. aeruginosa* Asp-tRNA. Lanes: 1, with AdT for 30 min; 2, with AdT for 15 min; 3, without AdT. These panels show phosphorimages of TLC of the ^{14}C-labeled amino acids after their removal from these tRNAs (see Materials and Methods) or free glutamine and glutamate as standards (panel A, lanes 3 and 4).

the biochemical evidence for the discriminating character of its GluRS (Fig. 6A). Moreover, structural evidence for this character comes from the presence of an Arg residue at the position corresponding to Arg 358 of domain 4 of *Thermus thermophilus* discriminating GluRS (GluRS-D) (Fig. 1), which interacts with the third nucleotide anticodon (C-36) of $tRNA^{Glu}$, and whose replacement by a Gln residue makes this a GluRS-ND (28). Finally, physiological evidence for the discriminating character of *P. aeruginosa* GluRS is that its production at a low level in *E. coli* JP 1449 allows this strain carrying a thermosensitive GluRS to grow at a restrictive temperature. On the other hand, stronger expression is toxic for this heterologous host (see above). The mechanism for the toxicity for *E. coli* of higher levels of this GluRS-D is unknown.

All of the characterized trimeric AdTs from microorganisms or organelles catalyze in vitro the transamidation of Glu-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ (10). Even in the organisms where only one of these substrates is present in vivo, the trimeric AdT has not lost the property of transamidating the other substrate provided by another organism, in vitro or in vivo (3, 5, 25). This is also the case for *P. aeruginosa* AdT, which finds in its host only Asp-$tRNA^{Asn}$ but which can transamidate *B. subtilis* Glu-$tRNA^{Gln}$ (Fig. 6B).

The C subunit of *T. thermophilus* AdT interacts weakly with the A and B subunits, and the A-B interaction is strong, as evidenced by the fact that only the A-B heterodimer was obtained at the end of several steps of purification from a crude extract of normal cells (2). In the *P. aeruginosa* AdT, there is also a weak interaction between the C and A subunits, since following the overproduction of the His-tagged C subunit together with the untagged A subunit (Fig. 3B), the only peptide retained on the affinity column was the C subunit (see above). To purify this enzyme by affinity chromatography, we overproduced the His-tagged C and B subunits together with the untagged A subunit (Fig. 3C), counting on the strong A-B interaction to retain A on the column. This strategy led to a pure and active AdT, with stoichiometric amounts of the three subunits (Fig. 4B).

In some organisms lacking an *asnA* or *asnB* gene encoding asparagine synthetase, AdT participates in asparagine biosynthesis via Asp-$tRNA^{Asn}$ transamidation (21, 30). As *P. aeruginosa* has an *asnB* gene, the only function of AdT in its host appears to be its participation in the formation of Asn-$tRNA^{Asn}$ involved in ribosomal protein biosynthesis.

AdT is present in all archaea, most bacteria, and all known organelles, with the exception of *Leishmania tarentolae* mitochondria (22). On the other hand, it is absent from the cytoplasm of eukaryotes, so inhibitors of bacterial AdT are expected to have a low toxicity. Therefore, the essential role of AdT reported here for the formation of Asn-tRNA in *P. aeruginosa* identifies this enzyme as a potential target for antibiotics to be designed against this and other pathogenic gram-negative bacteria that lack *asnS*, such as *Moraxella*, *Neisseria*, and *Burkholderia*, and all gram-positive bacteria that lack *glnS*.

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