

# Transfer RNA-dependent amino acid biosynthesis: An essential route to asparagine formation

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**Biochemical experiments and genomic sequence analysis showed that *Deinococcus radiodurans* and *Thermus thermophilus* do not possess asparagine synthetase (encoded by *asnA* or *asnB*), the enzyme forming asparagine from aspartate. Instead these organisms derive asparagine from asparaginyl-tRNA, which is made from aspartate in the tRNA-dependent transamidation pathway [Becker, H. D. & Kern, D. (1998) *Proc. Natl. Acad. Sci. USA* 95, 12832–12837; and Curnow, A. W., Tumbula, D. L., Pelaschier, J. T., Min, B. & Söll, D. (1998) *Proc. Natl. Acad. Sci. USA* 95, 12838–12843]. A genetic knockout disrupting this pathway deprives *D. radiodurans* of the ability to synthesize asparagine and confers asparagine auxotrophy. The organism's capacity to make asparagine could be restored by transformation with *Escherichia coli* *asnB*. This result demonstrates that in *Deinococcus*, the only route to asparagine is via asparaginyl-tRNA. Analysis of the completed genomes of many bacteria reveal that, barring the existence of an unknown pathway of asparagine biosynthesis, a wide spectrum of bacteria rely on the tRNA-dependent transamidation pathway as the sole route to asparagine.**

**A**sparagine, one of the 21 cotranslationally inserted amino acids that make up proteins, is known to be synthesized from aspartate in an ATP-dependent amidation reaction (1). Two mechanistically distinct asparagine synthetases are known (2–4). The one encoded by *asnA* utilizes ammonia as amide donor, whereas the *asnB*-derived protein works with glutamine. These enzymes are present in organisms of all domains, the major one being asparagine synthetase B, which is encoded in different organisms by a small number of related genes. Both enzymes are well studied biochemically (5), and their crystal structures are known (6, 7). Until recently they were assumed to be the sole biosynthetic route to asparagine. However, *Thermus thermophilus* and *Deinococcus radiodurans* lack these enzymes; instead they employ a tRNA-dependent transamidation mechanism for conversion of aspartate to asparagine (8, 9).

Two routes of Asn-tRNA synthesis exist in *D. radiodurans* (Fig. 1). Similar to many bacteria, *Deinococcus* contains a tRNA-dependent two-step pathway of Asn-tRNA formation. In the first step a nondiscriminating aspartyl-tRNA synthetase (AspRS)2 generates the misacylated Asp-tRNA<sup>Asn</sup> species, which then is amidated to the correctly charged Asn-tRNA<sup>Asn</sup> by the heterotrimeric Asp-tRNA<sup>Asn</sup> amidotransferase (Asp-AdT; encoded by the *gatCAB* genes) with glutamine serving as the amide donor (9). In addition, the organism also contains asparaginyl-tRNA synthetase (AsnRS; ref. 10), which is active and produces Asn-tRNA in the canonical aminoacylation reaction (9). The close *Deinococcus* relative *T. thermophilus* has similar enzymes and presumably uses the same asparagine biosynthetic routes (8, 11, 12). It was suggested earlier (8, 9) that the role of Asp-AdT in *D. radiodurans* and *T. thermophilus* is to synthesize the cell's entire supply of asparagine, because no *asnA* or *asnB* orthologs are present in the genome (10), and because biochemical analysis of crude cell extracts did not reveal the presence of any tRNA-independent asparagine synthetase activity (8, 9). Here we present data from *D. radiodurans* that prove this role to be correct and propose that tRNA-dependent asparagine syn-

thesis occurs in many bacteria as the sole synthetic route to this essential amino acid.

## Materials and Methods

**Chemicals and Enzymes.** Oligonucleotides were synthesized, and PCR products were sequenced by the Keck Foundation Biotechnology Resource Laboratory at Yale University. Uniformly labeled [<sup>14</sup>C]asparagine [228.4 mCi/mmol (1 Ci = 37 GBq)] was from NEN Life Science Products. Uniformly labeled [<sup>14</sup>C]aspartate (213 mCi/mmol) and [<sup>3</sup>H]aspartate (28 mCi/mmol) were from Amersham Pharmacia.

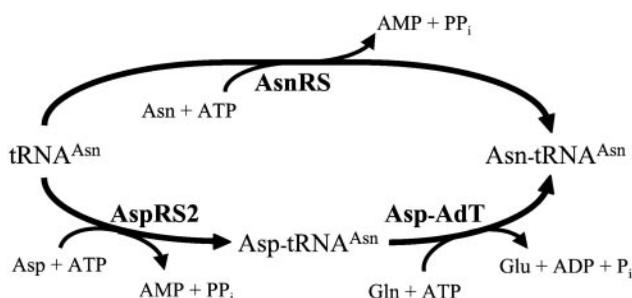
**Strains, Plasmids, and Bacterial Growth.** *D. radiodurans* strain R1 was obtained from John Battista (Louisiana State University, Baton Rouge, LA). Plasmids pMD66 (13) and pMD405 were kindly provided by Michael J. Daly (Uniformed Services University of the Health Sciences, Bethesda, MD). pMD66 replicates autonomously in both *Escherichia coli* and *D. radiodurans*. pMD405 contains ampicillin-resistance and kanamycin-resistance markers and an origin of replication for *E. coli* but no replication capacity for *D. radiodurans*. *D. radiodurans* was grown at 30°C with vigorous shaking or on medium containing 1.5% agar. Complex medium for *D. radiodurans* was TGY (0.8% tryptone/0.4% yeast extract/0.1% glucose). Minimal medium for *D. radiodurans* (14) was 20 mM potassium phosphate (pH 8.0)/0.2 mM MgCl<sub>2</sub>/0.1 mM CaCl<sub>2</sub>/5 μM manganese acetate/5 μM (NH<sub>4</sub>)<sub>2</sub>Mo<sub>7</sub>O<sub>24</sub>/5 μM FeSO<sub>4</sub>/10 μg/ml methionine/25 μg/ml histidine/30 μg/ml cysteine/1 μg/ml nicotinic acid/2 mg/ml fructose. Where necessary, the medium was supplemented with 10 μg/ml kanamycin/2.5 μg/ml tetracycline/3 μg/ml chloramphenicol/20 μg/ml asparagine. *E. coli* strain DH5α was grown at 37°C on LB medium (1% tryptone/0.5% yeast extract/0.5% NaCl/1.5% agar) supplemented where necessary with 50 μg/ml ampicillin and 30 μg/ml tetracycline. *E. coli* strain JF448 (15), obtained from the *E. coli* Genetic Stock Center at Yale University, has an asparagine auxotrophic phenotype (*asnA* and *asnB*). It was grown for 3 days at 37°C on M9 minimal agar plates containing 1 μg/ml thiamine and 30 μg/ml asparagine.

**Preparation of tRNA Transcripts.** tRNA<sup>Asp</sup> and tRNA<sup>Asn</sup> genes were identified in the preliminary *D. radiodurans* genomic DNA sequence by the program TRNASCAN (16). The tRNA<sup>Asp</sup> gene was produced by PCR using the *Pfu* Turbo polymerase (Stratagene) and subcloned into pUC119. The tRNA<sup>Asn</sup> gene was constructed in pUC119 from six overlapping oligonucleotides. To enable *in vitro* transcription of tRNA<sup>Asn</sup> with T7 RNA polymerase, the first base pair was changed to G1-C72. The 5' end of each construct contained the T7 promoter, and the 3' end contained a *Bst*NI site

Abbreviations: AspRS, aspartyl-tRNA synthetase; Asp-AdT, Asp-tRNA<sup>Asn</sup> amidotransferase; AsnRS, asparaginyl-tRNA synthetase.

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**Fig. 1.** Redundant pathways of Asn-tRNA<sup>Asn</sup> synthesis in *D. radiodurans*. Shown are the direct pathway (top half of diagram) and the transamidation pathway (bottom half of diagram).

that, after *Bst*NI digestion, allowed generation of the correct 3' end of the DNA template for *in vitro* transcription. Reactions were performed as described (17); the resulting RNA was ethanol-precipitated, resuspended in loading buffer [10 mM Hepes (pH 7.3)/1 mM Na<sub>2</sub>-EDTA/7 M urea], and heated to 85°C for 10 min before loading onto a Q Sepharose column (Amersham Pharmacia). Transcripts were eluted from the column at 1.3 M NaCl. Fractions were concentrated, desalted, ethanol-precipitated, and resuspended in 10 mM Hepes (pH 7.2).

**Preparation of Unfractionated and *in Vivo* Expressed tRNA.** Unfractionated *D. radiodurans* tRNA was prepared as described (9). For generation of *in vivo* expressed *D. radiodurans* tRNA<sup>Asn</sup>, the tRNA<sup>Asn</sup> gene was constructed from two overlapping oligonucleotides and cloned into the pGFIB vector (18). The plasmid was transformed into *E. coli* DH5 $\alpha$ , and unfractionated tRNA was prepared from cultures of these cells as described above (9). Comparison of aminoacylation reactions with this tRNA and unfractionated tRNA from DH5 $\alpha$  showed that the *D. radiodurans* tRNA<sup>Asn</sup> comprised 10–15% of the tRNA.

**Preparation of *D. radiodurans* Cell Extract.** *D. radiodurans* wild-type strain R1 and the deletion mutant strains were grown in TGY medium including kanamycin for the mutant strains. Cells (5 g) from logarithmic-phase growth were harvested by centrifugation at 4,000  $\times$  *g* for 10 min at 4°C and resuspended in 100 ml of ice-cold 95% ethanol to remove the cells' outer membrane. The ethanol-stripped cells were harvested immediately by centrifugation at 4,000  $\times$  *g* for 5 min at 4°C. The resulting pellets were resuspended in 5 ml of 25 mM Hepes (pH 7.2)/1 mM MgCl<sub>2</sub>/30 mM KCl/5 mM DTT/4 mM 2-mercaptoethanol/10% glycerol. Lysozyme (2 mg/ml) was added to the suspension, and the mixture was incubated on ice for 30 min. The cells were disrupted by sonication with a Branson Sonifier 250 at 60% output for 15 sec plus cooling on ice for 3 min, repeated 10–15 times. The disrupted cells were centrifuged at 100,000  $\times$  *g* for 2 h at 4°C. The extracts were dialyzed against the above buffer containing 50% glycerol and stored at –20°C.

**Preparation of *D. radiodurans* AspRS1 and AspRS2.** As described earlier (9), AspRS1 was overexpressed as a His-tagged enzyme from pET15b (Novagen) and purified on nickel-nitrilotriacetic acid resin (Qiagen, Chatsworth, CA). AspRS2 was overexpressed from pCYB2 (New England Biolabs) as a self-cleaving chitin-binding construct and purified on chitin agarose resin (New England Biolabs). To obtain cleavage of the intein fusion product, a glycine residue was added to the C terminus of AspRS2. The enzyme preparations were >95% pure as judged by Coomassie-stained polyacrylamide gel.

**Aminoacyl-tRNA Synthetase Assays.** Reactions (100  $\mu$ l) were performed as described (9) at 30°C and contained 100 mM Hepes (pH 7.2), 50 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 6  $\mu$ M [<sup>3</sup>H]aspartate, 2.5 mM unlabeled aspartate, 2 mM ATP, and 100 nM AspRS1 or 200 nM AspRS2. Aliquots (10  $\mu$ l) of the reaction were removed at various time intervals and spotted onto a 3MM filter disk (Whatman) that was immersed in 10% trichloroacetic acid for 10 min and then washed twice with 5% trichloroacetic acid. The filters were rinsed in ethanol, dried and counted in 3 ml of Ultima Gold scintillation mixture fluid (Packard).

For kinetic analysis, a higher specific activity aspartate mix was composed of 16  $\mu$ M [<sup>3</sup>H]aspartate and 2.5 mM unlabeled aspartate. Aliquots were taken every 15 sec, and initial rates were determined from a minimum of five time points in the linear range of duplications of the assay. The enzyme concentrations were held at 40 nM AspRS1 and 100 nM AspRS2. The concentrations of ATP and aspartate were at least 5-fold above their determined apparent *K<sub>m</sub>* values (2 mM ATP and 0.5 mM aspartate in the AspRS1 reactions, and 2 mM ATP and 1.25 mM to 2.5 mM aspartate for AspRS2). When tRNA<sup>Asn</sup> was used, the *K<sub>m</sub>* of AspRS2 for aspartate was 2-fold higher than that when tRNA<sup>Asp</sup> was used. Therefore, for AspRS2, aspartate was kept at 1.25 mM for tRNA<sup>Asp</sup> and 2.5 mM for tRNA<sup>Asn</sup>. Transcript concentrations were at 3  $\mu$ M for AspRS1 and 20  $\mu$ M for AspRS2. For AspRS1, the concentration of aspartate was varied from 2 to 250  $\mu$ M, ATP was varied from 0.05 to 5 mM, and tRNA<sup>Asp</sup> transcript was varied from 0.01 to 0.5  $\mu$ M. For AspRS2, aspartate was varied from 0.02 to 1.25 mM for tRNA<sup>Asp</sup> and 0.02 to 2.5 mM for tRNA<sup>Asn</sup>; ATP was varied from 13  $\mu$ M to 1.6 mM; tRNA<sup>Asp</sup> transcript was varied from 0.04 to 5  $\mu$ M; and tRNA<sup>Asn</sup> transcript was varied from 0.08 to 20  $\mu$ M.

**Preparation of Genomic DNA.** *D. radiodurans* genomic DNA was prepared as described (19) with modifications. Cells from a 500-ml culture were collected by centrifugation at 4°C for 15 min at 5,000  $\times$  *g*. The cell pellet was resuspended in 2 ml of 100% ethanol to facilitate the removal of lipid-rich outer membranes. The stripped cells were collected by centrifugation and resuspended in water. Lysozyme (4 mg) was added and the suspension incubated at 37°C for 30 min. SDS (2%), Na<sub>2</sub>-EDTA [0.1 M (pH 8.0)], and Pronase E (4 mg) were added, and the lysate was incubated at 50°C for 3 h. After removal of proteins by multiple extractions with phenol/chloroform/isoamyl alcohol (25:24:1), chromosomal DNA was recovered from the aqueous layer by ethanol precipitation. The DNA was resuspended in 10 mM Tris-HCl (pH 8.0).

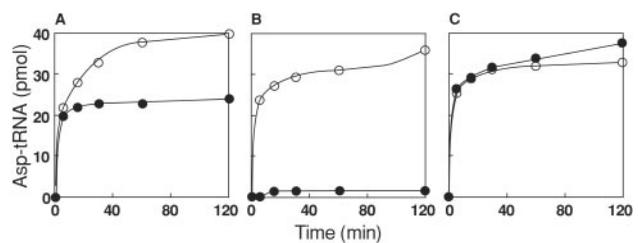
**Construction of an Artificial Operon for Asparagine Synthesis with *D. radiodurans* aspS2 and gatCAB genes.** To construct an artificial operon for asparagine synthesis, the *Nde*I/*Bam*HI fragment containing the *D. radiodurans* *gatCAB* genes from the pET20b clone (9) was ligated into the pCYB1 vector (New England Biolabs) such that there was no protein fusion with the intein of pCYB1. This process yielded the vector pCYB1-DR*gatCAB*. The *D. radiodurans* *aspS2* gene subsequently was cloned into this vector by PCR-amplifying *aspS2* with a forward primer containing the *Nde*I recognition site and a reverse primer containing the ribosome binding sequence for the *gatC* gene plus an *Ase*I recognition site. The amplified *aspS2* gene was cloned into the pCR-TOPO vector (Invitrogen), and the *Nde*I/*Ase*I fragment containing *aspS2* was subcloned into the *Nde*I site of pCYB1-DR*gatCAB*, yielding pCYB1-DR*aspS2gatCAB*.

**Construction of Deletion Mutants.** *D. radiodurans* strains with deletions of *asnS* (encoding AsnRS) or *aspS2* (encoding AspRS2) were constructed by an inversion overlap extension PCR knockout method. By using the Expand Long Template PCR system (Roche), for each gene deletion two PCR products were generated from *D.*

*radiodurans* genomic DNA based on the known sequence (10). PCR with primers A and B yielded the upstream region of the gene including the 5' end of *asnS* or *aspS2*, generating products of 3.0 or 2.7 kb, respectively. PCR with primers C and D similarly yielded the downstream region including the 3' end of *asnS* or *aspS2*, generating products of 3.0 or 2.7 kb, respectively. The 5' ends of primers A and D additionally contained complementary forms of the sequence 5'-GCGGCCGCGTTTAAACGGCGCGCC-3', which is comprised of the adjacent 8-bp restriction sites *NotI*, *PmeI*, and *AscI*, to aid in subsequent clone identification by restriction analysis. This sequence also allowed overlap extension PCR using the two PCR products above as template plus primers E and F. Primers E and F contained 5'-*XbaI* sites for cloning, overlapped the genomic sequence immediately outside of the gene, and were on the 3' side of primers B and C, respectively. The PCR products generated with primers E and F contained 54 or 71 bp of the 5' end of *asnS* or *aspS2*, respectively, and 57 or 72 bp of the 3' end of *asnS* or *aspS2*, respectively. Overlap PCR generated an  $\approx 6$ -kb fragment simultaneously linking and inverting the upstream and downstream regions of the gene. The resulting fragment was cloned into the *XbaI* site of plasmid pMD405 for amplification in *E. coli*. *D. radiodurans* was transformed (20) with these plasmids and plated on TGY medium plus kanamycin. A double-crossover recombination into the chromosome is expected to delete the gene without disrupting its flanking regions. The crossover event was verified in kanamycin-resistant transformants by PCR analysis. The AsnRS activity was verified in *D. radiodurans* cell extracts.

**Complementations of *D. radiodurans* with *E. coli* *asnB* and *E. coli* JF448 with the Artificial Operon for Asparagine Synthesis.** The *asnB* gene encoding asparagine synthetase was amplified from *E. coli* W3110 genomic DNA and cloned into the *ScaI* site of pMD66. This shuttle vector confers ampicillin resistance in *E. coli* and kanamycin and tetracycline resistance in *D. radiodurans*. Transformation of *D. radiodurans* was carried out as described (20). pCYB1, pCYB1-DR*aspS2*, pCYB1-DR*gatCAB*, and pCYB1-DR*aspS2gatCAB* were transformed into JF448. Ampicillin-resistant colonies were streaked onto M9 minimal agar plates containing 1  $\mu$ g/ml thiamine and 1 mM isopropyl  $\beta$ -D-thiogalactoside with or without 30  $\mu$ g/ml asparagine and incubated at 37°C for 3 days.

**Phylogenetic Analysis.** For the protein sequence alignments the following amino acid sequences for AsnB proteins and their homologs were obtained from the nonredundant protein database at the National Center for Biotechnology Information: *Aedes aegypti* (gb|AAB95197.1), *Aeropyrum pernix* (dbj|BAA81051.1), *Arabidopsis thaliana* (sp|P49078), *Archaeoglobus fulgidus* (gb|AAB89808.1), *Asparagus officinalis* (sp|P31752), *Bacillus halodurans* (dbj|BAB05227.1), *Bacillus subtilis* (sp|P54420, sp|P42113, and sp|O05272), *Bordetella bronchiseptica* (emb|CAA07658.1), *Caenorhabditis elegans* (gil|7438080 and emb|CAA92825.1), *Corynebacterium glutamicum* (dbj|BAA89484.1), *Desulfovibrio gigas* (gb|AAF34252.1), *Drosophila melanogaster* (gb|AAF45462.1), *E. coli* (sp|P22106), *Halobacterium* sp. (gil|10580431), *Homo sapiens* (sp|P08243 and ref|NP\_061921.1), *Methanothermobacter thermoautotrophicus* (gb|AAB84920.1), *Methanococcus jannaschii* (sp|Q58516), *Mycobacterium tuberculosis* (sp|Q10374), *Plasmodium falciparum* (gil|7494204), *Pseudomonas aeruginosa* (gb|AAG05472.1, gb|AAG03441.1, and gb|AAG06847.1), *Pyrobaculum aerophilum* (gb|AAL62750.1), *Pyrococcus abyssi* (emb|CAB50044.1 and emb|CAB50067.1), *Pyrococcus horikoshii* OT3 (dbj|BAA30201.1), *Saccharomyces cerevisiae* (sp|P49089, sp|P49090, and sp|Q04489), *Schizosaccharomyces pombe* (gil|7492408), *Thermoplasma acidophilum* (emb|CAC11460.1), *Vibrio cholerae* (gb|AAC46243.1), and *Xylella fastidiosa* (gb|AAF82931.1). Data from partial genome sequences were ob-



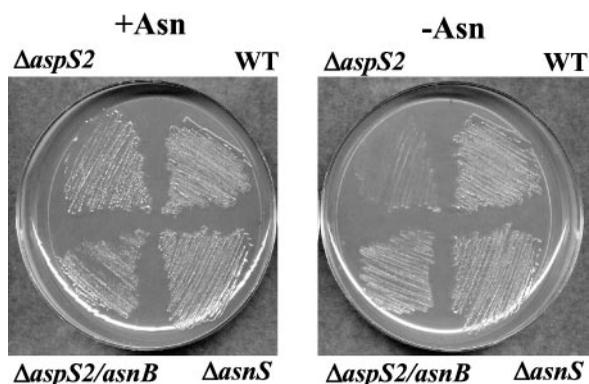
**Fig. 2.** Asp-tRNA formation by the two *D. radiodurans* AspRS enzymes. AspRS1 (●) and AspRS2 (○) were used to aminoacylate, per reaction, unfractionated *D. radiodurans* tRNA (A, 800 pmol), *D. radiodurans* tRNA<sup>Asn</sup> transcript (B, 40 pmol), and *D. radiodurans* tRNA<sup>Asp</sup> transcript (C, 40 pmol).

tained from: *Bacillus stearothermophilus*, www.genome.ou.edu/bstearo.html; *Giardia lamblia*, hermes.mbl.edu/baypaul/Giardia-HTML; *Acidithiobacillus ferrooxidans*, *Geobacter sulfurreducens*, and *Shewanella putrefaciens*, www.tigr.org; *Pyrococcus furiosus*, www.genome.utah.edu; and *Clostridium difficile*, *Streptomyces coelicolor*, and *Candida albicans*, www.sanger.ac.uk. Sequence data from *Prostheco bacter* sp. and *Bifidobacterium longum* were provided by R. Overbeek (Integrated Genomics, Chicago) and F. Arigoni (Nestlé Research Center, Lausanne, Switzerland), respectively.

**Phylogenetic Inference.** From the alignment of 50 AsnB proteins, 408 positions were deemed to be aligned confidently. These data were analyzed as described (21) by protein maximum parsimony methods using a heuristic search algorithm (PAUP\* 4.0 BETA 2, D. Swofford, Sinauer, Sunderland, MA). The 1,000 shortest trees were evaluated by maximum likelihood criteria, using the PROTML program (Version 2.2) in the MOLPHY package (22) with the JTT model for amino acid substitutions. Bootstrap percentages for each node in the tree were estimated by the resampling estimated log-likelihood method (23) using the PROTML program to compare the 1,000 most parsimonious trees. The CONSENSE program [PHYLP (phylogeny inference package), Version 3.5; J. Felsenstein, Department of Genetics, University of Washington, Seattle] was used to construct a consensus tree from the resampling estimated log-likelihood weightings. Phylogenetic trees were viewed and edited with the TREEVIEW program (Version 1.5.2; ref. 24).

## Results

***D. radiodurans* Contains Two AspRSs.** As discussed earlier (9) *Deinococcus* employs two routes of Asn-tRNA synthesis: canonical aminoacylation and tRNA-dependent transamidation (depicted in Fig. 1). A key enzyme for the latter pathway is a nondiscriminating AspRS capable of generating the misacylated Asp-tRNA<sup>Asn</sup> transamidation substrate. Actually, *D. radiodurans* contains two AspRS enzymes (9, 10). AspRS1 is a discriminating synthetase only capable of acylating the tRNA<sup>Asp</sup> isoacceptors, whereas the smaller, archaeal genre AspRS2 enzyme has relaxed tRNA specificity and aspartylates both tRNA<sup>Asp</sup> and tRNA<sup>Asn</sup> (9, 11, 12, 25, 26). To investigate the biochemical properties of these enzymes, they were purified from heterologously overexpressed cloned genes, and their activity was tested with *D. radiodurans* tRNA<sup>Asp</sup> and tRNA<sup>Asn</sup> species (Fig. 2). AspRS1 aminoacylated 3.4% of unfractionated tRNA from *D. radiodurans*. In contrast, AspRS2 aminoacylated a higher proportion ( $\approx 5\%$ ) of this tRNA (Fig. 2A), which is consistent with an ability to aminoacylate additional tRNA species. To determine whether the difference was caused by misaminoacylation of tRNA<sup>Asn</sup>, we assayed the ability of each AspRS to aminoacylate specific tRNA gene transcripts. Fig. 2B shows that only AspRS2 could aminoacylate the tRNA<sup>Asn</sup>, whereas both AspRS enzymes were fully capable of aminoacylating the tRNA<sup>Asp</sup> transcript (Fig. 2C).



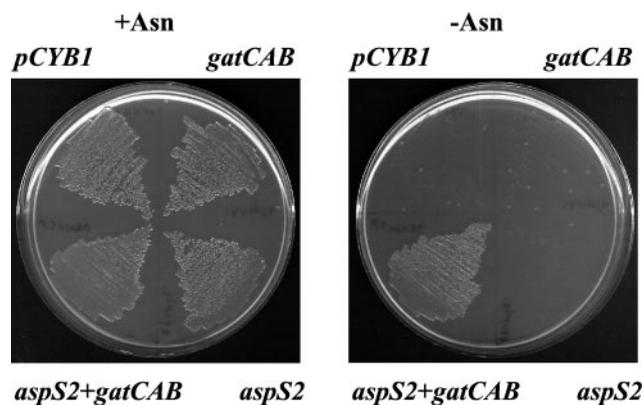
**Fig. 3.** Growth properties of *D. radiodurans* strain constructs.  $\Delta aspS2$ ,  $aspS2$  deletion strain; WT, wild-type parent strain;  $\Delta aspS2/asnB$ ,  $aspS2$  deletion strain complemented *in trans* with *E. coli asnB*;  $\Delta asnS$ ,  $asnS$  deletion strain. Growth on minimal agar plates in the presence (+Asn) or absence (–Asn) of asparagine is shown.

Detailed steady-state kinetic analysis with the tRNA<sup>Asn</sup> and tRNA<sup>Asp</sup> transcripts showed that the  $K_m$  of AspRS1 for the tRNA<sup>Asp</sup> transcript was 0.22  $\mu$ M, which is similar to the value of the *E. coli* enzyme for the homologous substrate of 0.6  $\mu$ M (27). The  $K_m$  of AspRS2 for each transcript was higher than for AspRS1 at 3.1  $\mu$ M for tRNA<sup>Asp</sup> and 1.8  $\mu$ M for tRNA<sup>Asn</sup>. The comparable  $K_m$  values of AspRS2 for both tRNA<sup>Asp</sup> and tRNA<sup>Asn</sup> demonstrates that AspRS2 recognizes both tRNAs to a similar degree. The  $K_m$  values for the other substrates are ATP (AspRS1), 216  $\mu$ M; ATP (AspRS2), 171  $\mu$ M (with tRNA<sup>Asp</sup>) and 24  $\mu$ M (with tRNA<sup>Asn</sup>); Asp (AspRS1), 69  $\mu$ M; and Asp (AspRS2) 247  $\mu$ M (with tRNA<sup>Asp</sup>) and 442  $\mu$ M (with tRNA<sup>Asn</sup>). These data are in general agreement with values for the two *T. thermophilus* AspRS enzymes (12, 28).

**Two Functionally Redundant Routes of Asn-tRNA Synthesis in *D. radiodurans*.** To demonstrate that two routes to Asn-tRNA formation exist in *D. radiodurans*, we attempted to create independent genetic knockouts of each pathway. The *asnS* gene of the published *D. radiodurans* R1 genomic sequence was reported to have an authentic frameshift (10); the predicted protein of 320 aa is not expected to be active. However, when we sequenced the *asnS* gene amplified from our isolate of *D. radiodurans* R1 DNA, we found an intact ORF of 521 aa in length. In agreement with this finding, we previously demonstrated AsnRS activity in a cell extract from this *Deinococcus* isolate (9).

We then proceeded to make two *D. radiodurans* strains with gene disruptions in either *asnS* (blocking aminoacylation, Fig. 1) or  $aspS2$  (blocking the transamidation pathway, Fig. 1). Both strains were found to be viable (Fig. 3 Left). Therefore, *D. radiodurans* possesses redundant routes to Asn-tRNA formation, and each can support the organism's growth independently.

**tRNA-Dependent Transamidation Is the Sole Route to Asparagine Formation in *D. radiodurans*.** The *D. radiodurans* genomic sequence has no apparent homologs of the *asnA* or *asnB* genes that encode the two types of asparagine synthetase (10). In accordance with this absence, asparagine synthetase activity could not be detected in cell extracts of *D. radiodurans* (9). Because there are no other known biosynthetic routes to asparagine (29), we reasoned that the tRNA-mediated transamidation pathway of Asn-tRNA synthesis is the route to asparagine formation in this organism. Thus, if this pathway is blocked by the  $aspS2$  deletion, the resulting *D. radiodurans* strain should be auxotrophic for



**Fig. 4.** Coexpression of *D. radiodurans aspS2* and *gatCAB* genes rescues asparagine auxotrophy of *E. coli* strain JF448. Growth on minimal agar plates in the presence (+Asn) or absence (–Asn) of asparagine is shown. The complementing genes were introduced on the pCYB1 vector.

asparagine. This phenotype of asparagine-dependent growth was verified on minimal medium in which the mutant did not grow without asparagine (Fig. 3 Right) but did grow in the presence of asparagine (Fig. 3 Left). In contrast, both the wild type and the *asnS* deletion mutant strains grew in the presence or absence of asparagine (Fig. 3). The *asnS* mutant does not require asparagine, because it forms the amino acid in a tRNA-dependent manner via the AspRS2/Asp-AdT transamidation pathway (Fig. 1). To demonstrate further that the  $aspS2$  deletion mutant requires asparagine, the strain was complemented *in trans* with the *E. coli asnB* gene (carried on a pMD66 plasmid). Similar to the wild type and the *asnS* deletion mutant, the  $aspS2$  strain complemented with *asnB* did not require added asparagine for growth (Fig. 3). These results show that *D. radiodurans* forms asparagine only in a tRNA-dependent manner.

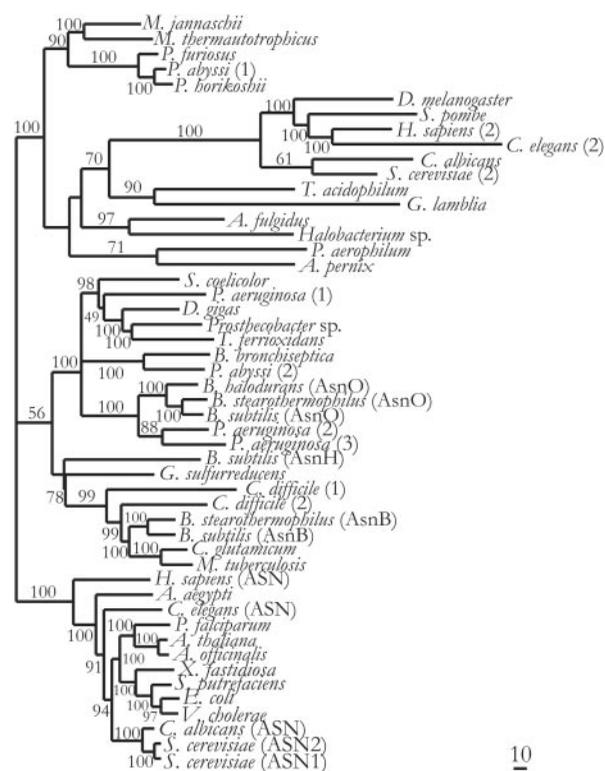
The end-product of the transamidation pathway is Asn-tRNA. Thus, if free asparagine is needed by the cell, Asn-tRNA must be hydrolyzed. Enzymes carrying out this function may exist, analogous to the protein that releases D-tyrosine from charged tRNA (30). Alternatively, AsnRS may fill this role, because synthetase-catalyzed hydrolysis of aminoacyl-tRNA in the absence of the other substrates (AMP and PP<sub>i</sub>) is known (31). The fact that the *D. radiodurans asnS* deletion grows in the absence of asparagine (Fig. 3 Right) shows that AsnRS does not assume this role in *Deinococcus*.

**The *Deinococcus* Transamidation Pathway Enzymes Rescue an *E. coli* Asparagine Auxotroph.** To demonstrate in another way that the transamidation pathway provides asparagine, we complemented the asparagine auxotrophic *E. coli* strain JF448 (*asnA,asnB*) with the *D. radiodurans aspS2, gatC, gatA, and gatB* genes. The transformed strain did not require asparagine for growth, unlike JF448 transformed with the empty vector or the  $aspS2$  or *gatCAB* genes (Fig. 4). Thus, the *Deinococcus* nondiscriminating AspRS2 and the Asp-AdT enzymes are functional in asparagine synthesis in *E. coli*.

**Two Pathways of Asparagine Formation Exist in Nature.** Based on the *Deinococcus* data presented above and assuming that there is no other still unknown pathway for asparagine synthesis, we examined bacteria with completed genome sequences for their possible asparagine biosynthetic mechanism. This analysis revealed that a large number of bacteria may use tRNA-dependent transamidation exclusively to generate asparagine. Table 1 shows that bacteria (with completed genome sequences) of 13 different

**Table 1. Presence (dark shadowed) of asparagine pathways among bacterial genera with complete (and incomplete) genomes**

	<i>gatCAB</i>	<i>asnA</i>	<i>asnB</i>	<i>asnS</i>
<b>Aquificales</b>				
<i>Aquifex</i>				
<b>CFB/Green sulfur bacteria</b>				
<i>Chlorobium</i>				
<b>Chlamydiales/ Verrucomicrobia</b>				
<i>Chlamydia</i>				
<i>(Prosthecoacter)</i>				
<b>Cyanobacteria</b>				
<i>Prochlorococcus</i>				
<i>Synechocystis</i>				
<i>Nostoc</i>				
<i>(Synechococcus)</i>				
<b>Flexibacter/Cytophaga/Bacteroides</b>				
<i>Porphyromonas</i>				
<i>(Cytophaga)</i>				
<i>(Bacteroides)</i>				
<b>Fusobacteria</b>				
<i>(Fusobacterium)</i>				
<b>Gram-positive bacteria, high G+C</b>				
<i>Bifidobacterium longum</i>				
<i>(Thermobifida)</i>				
<i>Corynebacterium</i>				
<i>Mycobacterium</i>				
<i>Streptomyces</i>				
<b>Gram-positive bacteria, low G+C</b>				
<i>Mycoplasma</i>				
<i>Staphylococcus</i>				
<i>Enterococcus faecalis</i>				
<i>Streptococcus</i>				
<i>Ureaplasma</i>				
<i>Bacillus</i>				
<i>Clostridium acetobutylicum</i>				
<i>Lactococcus</i>				
<b>Green non-sulfur bacteria</b>				
<i>(Chloroflexus)</i>				
<i>(Dehalococcoides)</i>				
<b>Heliobacteria</b>				
<i>(Desulfotubacterium)</i>				
<b>Proteobacteria, α-subdivision</b>				
<i>Agrobacterium</i>				
<i>Brucella</i>				
<i>Caulobacter</i>				
<i>Rickettsia</i>				
<i>Mesorhizobium</i>				
<i>(Magnetospirillum)</i>				
<b>Proteobacteria, β-subdivision</b>				
<i>Neisseria</i>				
<i>Bordetella</i>				
<i>(Nitrosomonas)</i>				
<b>Proteobacteria, γ-subdivision</b>				
<i>(Acidithiobacillus)</i>				
<i>Haemophilus</i>				
<i>Escherichia coli</i>				
<i>(Legionella)</i>				
<i>Yersinia</i>				
<i>Pseudomonas</i>				
<i>Vibrio</i>				
<i>Xylella</i>				
<b>Proteobacteria, δ-subdivision</b>				
<i>(Geobacter)</i>				
<i>Desulfovibrio</i>				
<b>Proteobacteria, ε-subdivision</b>				
<i>Campylobacter</i>				
<i>Helicobacter</i>				
<b>Spirochaetales</b>				
<i>Borrelia</i>				
<i>Treponema</i>				
<b>Thermotogales</b>				
<i>Thermotoga</i>				
<b>Thermus/Deinococcus</b>				
<i>Thermus</i>				
<i>Deinococcus</i>				



**Fig. 5.** Phylogeny of AsnB homologs. Bootstrap percentages for each branch were estimated by the resampling estimated log-likelihood method. (Scale bar represents 10 substitutions per 100 aa positions.)

genera, representing most of the main bacterial groups, have no apparent homologs for asparagine synthetase and AsnRS but contain the *gatCAB* genes encoding the bacterial Asp/Glu-tRNA amidotransferase. Therefore we propose that tRNA-dependent asparagine formation is widespread in the bacteria. The bacterial genomes also demonstrate that many other bacterial genera may make asparagine by the asparagine synthetase(s) of the AsnA or AsnB type (Table 1).

### Discussion

**Asparagine Biosynthesis Is Both tRNA-Dependent and tRNA-Independent.** The deletion experiments described above prove that *D. radiodurans* lacks a tRNA-independent route of asparagine biosynthesis. Unless there are yet undescribed biosynthetic routes to asparagine, one may conclude from existing genomic sequences and the established activities of the GatCAB enzyme that many bacteria synthesize asparagine only via this path (Table 1). Alternatively, some bacteria employ AsnRS to synthesize Asn-tRNA<sup>Asn</sup> with asparagine either imported or made from aspartate by the AsnA or AsnB enzymes (Table 1).

All the above pathways are distributed broadly among diverse bacteria (Table 1). However, some heterotrophic organisms such as *Porphyromonas gingivalis* apparently have no capacity for asparagine biosynthesis, relying solely on exogenous amino acids. The redundancy of asparagine biosynthetic pathways in bacteria epitomizes the modularity of both amino acid biosynthesis and tRNA-charging enzymes. As both evolution and genetics have demonstrated, these modular systems are interchangeable. Each system depends on compatible biosynthetic and tRNA-charging systems, emphasizing the unique role of aminoacyl-tRNA synthetases in connecting metabolism to translation.

**Evolution and Structural Relationships of the Enzymes of Asparagine Biosynthesis.** Most bacteria have some capacity to synthesize asparagine. The *asnA* gene has been identified primarily in pathogenic,

heterotrophic bacteria, many of which also have *asnB* genes (Table 1). A recent crystal structure of *E. coli* AsnA (6) supports the idea that *asnA* diverged from an AspRS gene (*aspS*). This model extends earlier sequence analyses (32) and site-directed mutagenesis experiments (33, 34) to explain how AsnA and AspRS form analogous aminoacyl-adenylate reaction intermediates (26, 35). Similarities between archaeal/eukaryal AspRS sequences and AsnA led to the suggestion that the *asnA* gene may have evolved specifically from an archaeal *aspS* gene (6).

Phylogenetic analysis of AsnB, the glutamine-dependent asparagine synthetase, shows homologs segregating into three distinct lineages: bacterial, eukaryal, and archaeal (Fig. 5). These results are consistent with AsnB evolving before the segregation of organisms into the modern cell types. AsnB shows no similarity to either AsnA or aminoacyl-tRNA synthetase proteins. Rather, its N-terminal domain resembles glutamine phosphoribosylpyrophosphate amidotransferase, whereas its C-terminal domain resembles GMP synthetase (7). Although eukaryotes depend on *asnB* homologs for asparagine biosynthesis, this gene does not seem to have evolved in bacteria as a primary route to make asparagine, because few bacteria seem to rely exclusively on the AsnB+AsnRS system for asparagine biosynthesis (Table 1). Remarkably, *asnB* genes have been identified in genomes of proteobacteria and archaea that have no recognizable AsnRS.

The aspartyl-tRNA<sup>Asn</sup> amidotransferase (GatCAB) is the most widespread route to asparagine synthesis in bacteria. Identification of tRNA-dependent asparagine biosynthesis by genomic analysis is complicated, however, by the fact that the aspartyl-tRNA<sup>Asn</sup> amidotransferase is one of two possible activities of the GatCAB enzyme. In most bacteria this amidotransferase also generates Gln-tRNA<sup>Gln</sup> employing the glutamyl-tRNA<sup>Gln</sup> activity. Whether an organism uses one or both of these activities is determined, in turn, by the presence of a nondiscriminating glutamyl-tRNA synthetase or AspRS in the genome (36). In any case, the absence of AsnRS in a genome suggests formation of Asn-tRNA<sup>Asn</sup> by the amidotransferase (e.g., *Chla-*

*mydia*; ref. 36). The amidotransferase pathway, including the nondiscriminating AspRS2, is therefore the sole route to translational asparagine biosynthesis in a majority of bacterial lineages (Table 1) and also in some archaeal lineages (37). As demonstrated here, GatCAB and AspRS2 are necessary and sufficient for asparagine biosynthesis in *D. radiodurans*. Furthermore, these genes are able to rescue asparagine auxotrophy (caused by *asnA*, *asnB*) in *E. coli*. It would be tempting to speculate that the amidotransferase pathway is the original “rational” route to asparagine, by forming directly Asn-tRNA, the required precursor for protein synthesis.

**A Wider Role for tRNA-Dependent Amino Acid Transformations.** Pathways whereby aminoacylated tRNAs are modified further before their use in translation are well known. Selenocysteinyl-tRNA (reviewed in ref. 38) and formylmethionyl-tRNA formation (reviewed in ref. 39) are crucial for translation in many organisms. Although the transamidation route to Asn-tRNA and Gln-tRNA synthesis is known to be operating in most bacteria and archaea, its wider significance in being responsible for an organism’s sole supply of asparagine has not been appreciated. Another example highlighting a close relationship between aminoacyl-tRNA synthesis and intermediary metabolism is the formation of 5-aminolevulinic acid, the universal precursor of porphyrins in many organisms. This metabolite is generated by reduction of glutamate attached to tRNA by a unique oxidoreductase with sequence-specific aminoacyl-tRNA recognition (40). These reactions suggest a closer than previously recognized tRNA-mediated connection between intermediary metabolism and protein synthesis.

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