

# Defining Sequence Space and Reaction Products within the Cyanuric Acid Hydrolase (AtzD)/Barbiturase Protein Family

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Cyanuric acid hydrolases (AtzD) and barbiturases are homologous, found almost exclusively in bacteria, and comprise a rare protein family with no discernible linkage to other protein families or an X-ray structural class. There has been confusion in the literature and in genome projects regarding the reaction products, the assignment of individual sequences as either cyanuric acid hydrolases or barbiturases, and spurious connection of this family to another protein family. The present study has addressed those issues. First, the published enzyme reaction products of cyanuric acid hydrolase are incorrectly identified as biuret and carbon dioxide. The current study employed <sup>13</sup>C nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry to show that cyanuric acid hydrolase releases carboxybiuret, which spontaneously decarboxylates to biuret. This is significant because it revealed that homologous cyanuric acid hydrolases and barbiturases catalyze completely analogous reactions. Second, enzymes that had been annotated incorrectly in genome projects have been reassigned here by bioinformatics, gene cloning, and protein characterization studies. Third, the AtzD/barbiturase family has previously been suggested to consist of members of the amidohydrolase superfamily, a large class of metallohydrolases. Bioinformatics and the lack of bound metals both argue against a connection to the amidohydrolase superfamily. Lastly, steady-state kinetic measurements and observations of protein stability suggested that the AtzD/barbiturase family might be an undistinguished protein family that has undergone some resurgence with the recent introduction of industrial *s*-triazine compounds such as atrazine and melamine into the environment.

With widespread DNA sequencing and complementary bioinformatics, more than 10<sup>8</sup> proteins are contained within the GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank/>). The proteins, both known and virtual, have been assigned to family and superfamily compilations. Some of these compilations now include tens or hundreds of thousands of members. Many have multiple crystal structures available (2–4, 14, 28). Newly identified proteins typically map to one of these large superfamilies, thereby aiding in determining the new protein's structure and function.

The set of proteins identified as cyanuric acid hydrolases and barbiturases do not fit this typical pattern. The cyanuric acid hydrolases designated AtzD and TrzD were the first members of this family to be identified (12, 20). The *atzD* gene in *Pseudomonas* sp. strain ADP is part of an operon that encodes the catabolism of the *s*-triazine compound cyanuric acid (13, 26). Cyanuric acid catabolism releases ammonia to support cell growth. AtzD initiates catabolism by opening the *s*-triazine ring to allow subsequent ammonia-liberating reactions. TrzD is found in *Acidovorax citrulli* strain NRRLB-12227, a bacterium isolated for its ability to grow on melamine. TrzD is functionally identical to AtzD, catalyzing the cyanuric acid ring-opening reaction in the pathway. AtzD and TrzD were identified as being homologous, but they were not connected to any other proteins or protein families in those studies (12, 20, 26). Barbiturase was first purified to homogeneity from *Rhodococcus erythropolis* JCM3132 and shown to catalyze the hydrolytic opening of barbituric acid, an intermediate in the oxidative pyrimidine catabolic pathway (34, 35). Those studies reported that barbiturase was homologous to AtzD and TrzD (34, 35). One of the reports found zinc to be present in the enzyme preparations and thus proposed that barbiturase was homologous to members of the amidohydrolase superfamily (35). Barbiturase is not reactive with cyanuric acid. Conversely, cyanuric acid hydrolase does

not hydrolyze barbituric acid, although that compound was observed to be a tight-binding inhibitor (11, 20).

Most recently, a few additional cyanuric acid hydrolases were experimentally confirmed in atrazine-degrading bacteria and from the genome sequence of *Moorella thermoacetica* ATCC 39073 (23). Additionally, several sequences have been annotated as cyanuric acid hydrolases or barbiturases from genome sequencing projects. Pairwise sequence comparisons of these new proteins typically show 40 to 65% protein sequence identity to AtzD, TrzD, or barbiturase, but all of these comparisons were similar. Thus, it was not possible to definitively discern whether a new sequence represented a cyanuric acid hydrolase, a barbiturase, or perhaps some other enzyme activity.

Cyanuric acid hydrolases and barbiturases, although clearly homologous, have been perceived as catalyzing somewhat different reactions. Barbiturase was reported to cleave a single amide bond in the six-membered ring of barbiturate to yield ureidomalononic acid (Fig. 1B). Purified cyanuric acid hydrolases have been reported by several groups (12, 20, 36) to yield biuret and carbon dioxide, which represents an apparent cleavage of two carbon to nitrogen bonds within the six-membered ring of cyanuric acid (Fig. 1A).

The present study dealt with these previous ambiguous features of

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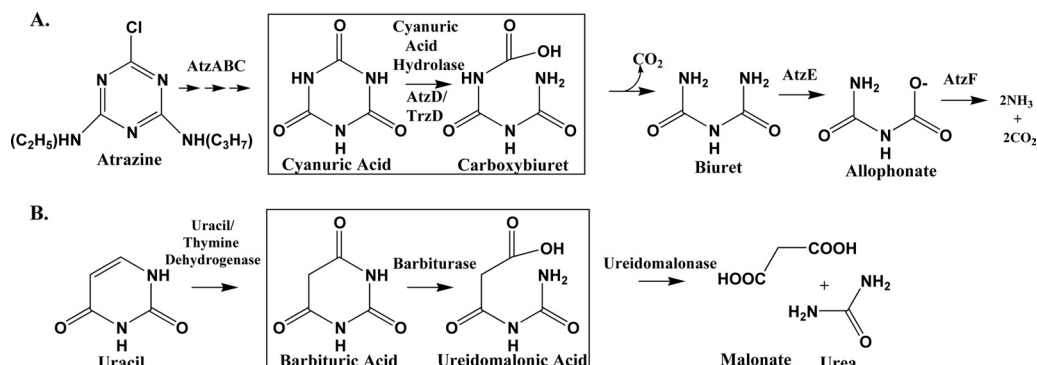


FIG 1 Metabolic pathways showing the reactions catalyzed by cyanuric acid hydrolase (A) and barbiturase (B).

the AtzD/barbiturase family and greatly expanded the set, with dozens of new related proteins being identified by bioinformatics. In total, 41 protein sequences were clustered into clades. Representative proteins from each clade were cloned, expressed, purified, and assayed to determine kinetic constants. This allowed assignment and, in a few cases, reassignment of proteins into clearly delineated classes as either cyanuric acid hydrolases or barbiturases. Moreover, the reactions catalyzed by cyanuric acid hydrolase and barbiturase were investigated and found to be directly analogous (Fig. 1). This correction of the reaction products is important because genomic and metabolism databases such as MetaCyc incorrectly describe the cyanuric acid hydrolase (AtzD and TrzD)-catalyzed reactions as amide hydrolysis and decarboxylation (<http://biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-5169>) rather than the amide bond cleavage reaction alone, as shown here. Lastly, bioinformatics and metal determinations showed no evidence of homology between the AtzD/barbiturase family and the amidohydrolase superfamily.

## MATERIALS AND METHODS

**Chemicals.** Cyanuric acid, barbituric acid, 2,4,5-trihydroxypyrimidine, 6-azauracil, and hydantoin were purchased from Acros Organics (Morris Plains, NJ). Uracil, thymine, 5-azacytosine, cytosine, 5-nitrobarbituric acid, 5,6-dihydroxyuracil, allantoin, L-asparagine, biuret, and deuterium oxide ( $\text{D}_2\text{O}$ ) were purchased from Sigma-Aldrich (St. Louis, MO). Creatinine was purchased from Fluka (Buchs, Switzerland). Alloxan was purchased from Eastman Kodak (Rochester, NY). Urea and dioxane were purchased from Fisher Scientific (Pittsburgh, PA). Ammelide and ammeline were synthesized in our laboratory as previously described (31).

**Identification of AtzD/barbiturase family members.** The nonredundant protein database was searched using pblast and PSI-BLAST with query sequences that included cyanuric acid hydrolase sequences from *Pseudomonas* sp. ADP (AtzD), *Acidovorax citrulli* strain 12227 (TrzD), and *Moorella thermoacetica* ATCC 39073 and the barbiturase sequence from *Rhodococcus erythropolis* JCM 3132. The data set was cross-referenced to ensure that all proteins were members of the same family. Shotgun analyses with both BLAST and PSI-BLAST were done in an attempt to broaden the sequence space of the AtzD/barbiturase family (30). The HMMER software (version 3.0) (9) was used to search the nonredundant protein database using Pfam hidden Markov models (HMMs), and HMMs were generated with the current data set.

**Phylogenetic tree construction.** The sequences belonging to the AtzD/barbiturase family were collected and aligned using ClustalW (37). Phylogenetic trees were constructed using Phylip software (10). Maximum-likelihood and neighbor-joining methods were used with bootstrapping. The protein from *Frankia* sp. strain Eul1c had the most divergent sequence and was used as the outgroup. Changing outgroups did not alter the morphology of the trees.

The general clusterings found in maximum-likelihood and neighbor-joining trees were the same. Slight differences in position were observed for AtzD homologs from *Bacillus cellulosilyticus* DSM 2522, *Paenibacillus* sp. strain JDR-2, *Acidithiobacillus ferrooxidans* ATCC 53993 and ATCC 23270, *Oceanicola granulosa* HTCC2516, *Saccharomonospora viridis* DSM 43017, and *Catenulispora acidiphila* DSM 44928. These differences did not alter interpretation of the data.

**Bacterial strains.** Strains were grown as recommended by the suppliers. *Rhizobium leguminosarum* bv. viciae 3841 was obtained from Peter Young (University of York, United Kingdom), *Azorhizobium caulinodans* ORS 571 was from Eric Giraud (Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France), *Moorella thermoacetica* was from Stephen Ragsdale (University of Michigan Medical School, Ann Arbor, MI), *Bradyrhizobium japonicum* USDA 110 was from the USDA-ARS *Rhizobium* culture collection in Beltsville, MD, and DNA from *Methylobacterium* sp. strain 4-46 was from Chris Marx (Harvard University, Cambridge, MA) and Lynne Goodwin (DOE-Joint Genome Institute, Los Alamos National Laboratory).

**Cloning, expression, and protein purification.** Genes encoding AtzD/barbiturase family members were PCR amplified and cloned into a pET28b+ vector (Novagen, Madison, WI), using NdeI and HindIII restriction sites. The resulting vectors were transformed into either *Escherichia coli* BL21(DE3) or *E. coli* BL21(DE3)pLys. These strains were grown with the appropriate antibiotics, i.e., 50  $\mu\text{g}/\text{ml}$  kanamycin or 50  $\mu\text{g}/\text{ml}$  kanamycin with 25  $\mu\text{g}/\text{ml}$  chloramphenicol, respectively. Expression and protein purification were conducted as previously described (23). All enzymes that were purified were stored at 4°C and never frozen, with the exception of the *Moorella thermoacetica* ATCC 39073 enzyme used in nuclear magnetic resonance (NMR) experiments. Stability was monitored to ensure that no detectable inactivation occurred throughout the study.

**Enzyme assays and kinetic constant determinations.** All reactions were carried out in 25 mM Tris-HCl buffer, pH 8.0, at room temperature unless otherwise noted. Assays were performed using a Beckman DU 640 spectrophotometer. Cyanuric acid and barbituric acid concentrations were monitored at 214 nm (extinction coefficient =  $8,800 \text{ cm}^{-1} \text{ M}^{-1}$ ) and 256 nm, respectively. Substrates for routine assays were tested at 100  $\mu\text{M}$ . Kinetic parameters were determined by obtaining rates at cyanuric acid concentrations ranging from 10 to 110  $\mu\text{M}$ . Kinetic constants were determined by using the nonlinear regression analysis of Prism software (GraphPad, version 5.03).

The *Azorhizobium caulinodans* ORS 571 (locus AZC\_3203) enzyme was tested with additional substrates, including uracil, thymine, 6-azauracil, 5-azacytosine, cytosine, 2,4,5-trihydroxypyrimidine, 5-nitrobarbituric acid, creatinine, 5,6-dihydroxyuracil, hydantoin, ammelide, ammeline, allantoin, alloxan, urea, L-asparagine, and biuret. Transformation of these compounds consisted of noting any spectral changes via UV-visible spectrometry and any changes in peak areas via high-pressure liquid chromatography (HPLC) analysis, using a Hewlett-Packard HP 1100 system

equipped with a diode array detector interfaced to an HP ChemStation. HPLC samples were prepared in 5 mM phosphate buffer, pH 7.0. Samples were analyzed on either an Alltech mixed-mode C<sub>8</sub>/anion 7  $\mu$  column (250 by 4.6 mm), using an isocratic mobile phase consisting of 95% methanol and 5% 5 mM phosphate buffer, pH 7.0; a Waters IC-Pak A HC anion-exchange column (150 by 4.6 mm), using an isocratic mobile phase consisting of 5 mM phosphate buffer, pH 8.0; or a Varian Microsorb-MV 100-8 CN column (250 by 4.6 mm), using an isocratic mobile phase of 5 mM phosphate buffer, pH 6.7.

**Demonstration of cyanuric acid hydrolase reaction product via <sup>13</sup>C NMR and mass spectrometry: detection of [U-<sup>13</sup>C]carboxybiuret.** Initially, broadband proton-decoupled <sup>13</sup>C NMR spectra were acquired at 20°C and 100.5 MHz on a Varian Unity Inova 400 MHz NMR spectrometer equipped with a Nolorac 4 Nuc probe and operating under VnmrJ 2.2D software. 1,4-Dioxane was used as internal reference and integration standard (assigned as  $\delta = 66.6$  ppm relative to an external tetramethylsilane reference). Initial <sup>13</sup>C NMR results were confirmed using a Varian Inova 600 MHz NMR spectrometer with a 5-mm Auto-X Dual Broadband probe held at 25°C.

Reactions were performed in 5-mm NMR tubes containing a 0.7-ml solution of uniformly ring-labeled [U-<sup>13</sup>C]cyanuric acid (2.0 mg/ml, 95% chemical purity, 99% isotopic purity; Toronto Research Chemicals, Inc., Ontario, Canada) in 100 mM potassium phosphate, 10% D<sub>2</sub>O, and 1% 1,4-dioxane (pH 8.9). The mixture had a final pH of  $\sim 7.7$ . The <sup>13</sup>C NMR spectrum of the [U-<sup>13</sup>C]cyanuric acid (singlet, 157.7 ppm) was stable overnight at room temperature. Spectra from commercial standards of biuret and bicarbonate were also acquired and had chemical shifts of 157.5 (singlet) and 160.4 ppm (singlet), respectively. Enzymatic reactions were initiated by adding 35 to 150  $\mu$ g purified cyanuric acid hydrolase in 100 mM potassium phosphate buffer (pH 7.0) to the NMR tube. The cyanuric acid hydrolases used were from *Pseudomonas* sp. ADP, *Moorella thermoacetica* ATCC 39073, and *Azorhizobium caulinodans* ORS 571 (locus AZC\_3892). Spectra representing the averaged chemical shifts from all pulses during the acquisition time were viewed at various intervals to follow the reaction process.

For mass spectrometry, 1-ml reaction mixtures contained 2.0 mg/ml cyanuric acid (unlabeled or U-<sup>13</sup>C labeled) in 100 mM ammonium acetate, pH 9.1. The pH with this mixture was 8.5. Reactions were initiated with the addition of 35  $\mu$ l cyanuric acid hydrolase in 100 mM potassium phosphate, pH 7. Twenty-microliter samples were directly injected into a Waters high-resolution mass spectrometer in negative-ion mode at intervals over the course of 40 min with 3 ml/min water as the carrier fluid. Spectra were analyzed using the MassLynx software with Elemental Composition. Mass determinations were made in the following manner. Each ion between nominal masses of 100 and 500 was analyzed for its abundance over time. Those masses with profiles that reflected increasing abundance and then subsequent decreasing abundance were noted. The associated masses from the experiment with unlabeled cyanuric acid were then compared to those from the experiment with [U-<sup>13</sup>C]cyanuric acid to generate a list of paired ions, where the difference between their masses could be attributed to the difference between <sup>12</sup>C and <sup>13</sup>C. For each of the paired ions selected, the average mass from each time point was averaged and the standard deviation determined (Microsoft Excel 2003) to obtain the mass and tolerance to use in the Elemental Composition software.

**Metal analysis.** The cyanuric acid hydrolases from *Pseudomonas* sp. ADP, *Moorella thermoacetica* ATCC 39073, and *Bradyrhizobium japonicum* USDA 110 and the barbiturase from *Rhodococcus erythropolis* JCM 3132 were hydrolyzed with 6 M hydrochloric acid at 110°C for 24 h under vacuum. After cooling, the samples were quantitatively transferred to a vial and diluted to 5 ml with Chelex-treated water. Metal concentrations were determined via inductively coupled plasma emission spectroscopy (ICP) at the Soil Analytical Laboratory of the University of Minnesota (St. Paul, MN). Protein concentrations were quantitatively ascertained by amino acid analysis at Purdue Proteomic Facility (West Lafayette, IN).

## RESULTS

**Identification of new members in the cyanuric acid hydrolase (AtzD)/barbiturase family.** Sequences from over 6,423 genomes (24) containing over 10 million genes were analyzed in an effort to expand the set of known cyanuric acid hydrolases/barbiturases and link them to other protein families using BLAST, PSI-BLAST, Shotgun, and HMMER. Efforts to link this family with another family or superfamily were unsuccessful. However, the effort did identify 41 homologous sequences as of 4 January 2011. Of those, 8 were identified in bacteria that metabolize *s*-triazine compounds, 32 were identified from genome sequencing data, and 1 was determined independently.

The cyanuric acid hydrolase/barbiturase proteins consist of 340 to 400 amino acids and have subunit molecular masses of 36 to 43 kDa. The sequence diversity was large, with most proteins 40 to 65% identical to any of the proteins of known function. The identified sequences were found in a diverse set of organisms that represented one eukaryotic (one member only) and five bacterial phyla. These organisms were found in a wide range of environments, which included soils, acid mine drainage, plant tissue, and human feces. Additionally, the source organisms had been studied for a diverse set of functions, including plant symbioses, photosynthesis, *s*-triazine degradation, and plant pathogenesis.

**Phylogenetic analysis of protein sequences.** Phylogenetic analysis of the cyanuric acid hydrolase/barbiturase sequences generated a tree in which 39 of the 41 sequences were placed into two major clades (Fig. 2). Clade 1 contained 24 sequences, of which 20 were subdivided into 3 groups (groups 1, 2, and 3) based on branching patterns. Clade 2 contained 15 sequences, of which 14 were organized into 2 groups (groups 4 and 5) based on branching patterns and gene context (see Fig. S1 in the supplemental material). Sequences that varied their position depending on the tree-building algorithm used were left out of the defined groups. Clade 1 sequences were more divergent than clade 2 sequences. Most of the clade 1 sequences were only  $\sim 40$  to 65% identical to known functional family members, whereas all of the clade 2 sequences were  $\geq 72\%$  identical to the characterized barbiturase (Table 1).

The placement of proteins in four of the five groups correlated with the phylogeny of the source organisms. Groups 1 and 3 were composed exclusively of proteins from the *Firmicutes* and *Alphaproteobacteria*, respectively. Groups 4 and 5 were composed exclusively of actinobacterial proteins. These observations suggested that members of the cyanuric acid hydrolase/barbiturase family may be ancient, divergent, and chromosomally encoded in many organisms. The last point is supported by the gene placement within sequenced genomes (see Fig. S1 in the supplemental material).

Group 2 sequences were from a diverse set of organisms, representing *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*. All but one of the group 2 source organisms were isolated for their *s*-triazine degradation abilities, and the sequences from those organisms are  $>99\%$  identical to either AtzD or TrzD (Fig. 2). Many of these genes have been associated with plasmids and transposons and could have spread to a variety of organisms via horizontal gene transfer (5, 7, 12, 26, 32). It is possible that some of these genes have spread more rapidly in recent times with the inadvertent selection pressure provided by the use of billions of pounds of anthropogenic *s*-triazine compounds.

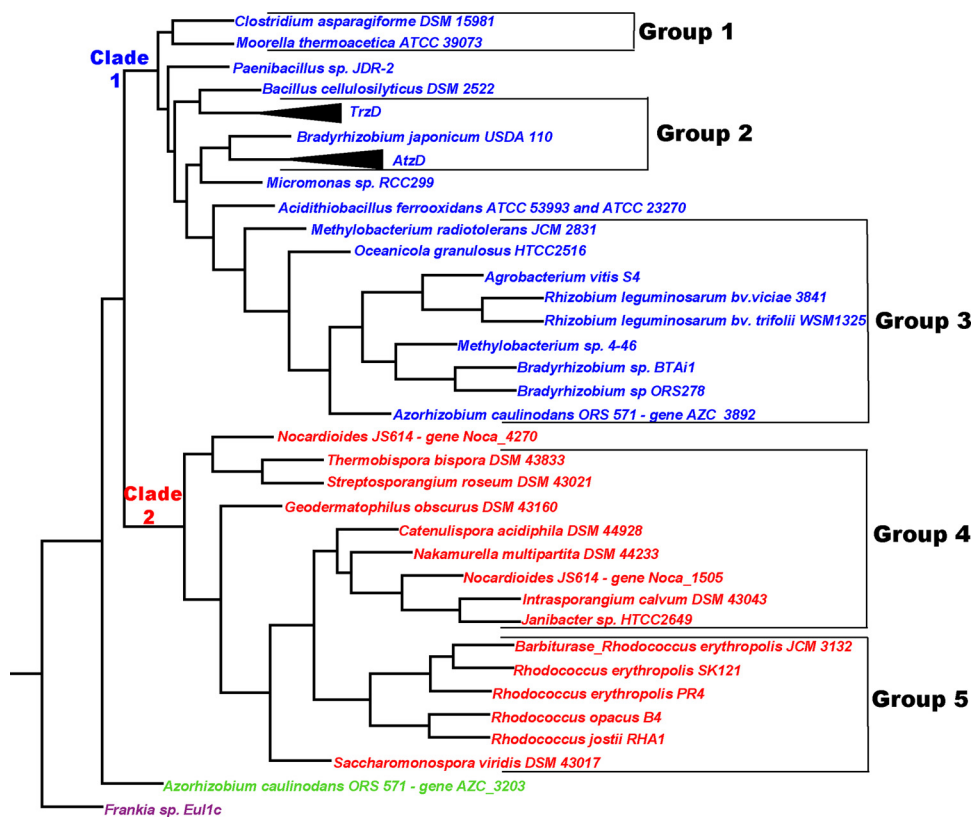


FIG 2 Maximum-likelihood phylogenetic tree for the AtzD/barbiturase family. Clade 1 (red), containing predicted cyanuric acid hydrolases, is divided into three groups: group 1, *Firmicutes* enzymes; group 2, enzymes found in phylogenetically diverse bacteria with *s*-triazine degradation abilities (proteins sharing >99% identity to either AtzD [*Pseudomonas* sp. ADP, *Pseudomonas nitroreducens*, *Comamonas* sp. strain A2, *Arthrobacter* sp. strain MCMB-436, and *Arthrobacter* AD25] or TrzD [*Acidovorax citrulli* strain 12227, *Enterobacter cloacae* strain 99, and *Aminobacter aminovorans*] have condensed subtrees); and group 3, alphaproteobacterial enzymes. Clade 2 enzymes (blue) are found in actinobacteria and are divided into two groups, based on gene context data (see Fig. S1 in the supplemental material): group 4, containing an adjacent amino acid kinase superfamily protein, and group 5, containing the experimentally determined barbiturase from *Rhodococcus erythropolis* JCM 3132 and with an amidase signature superfamily member adjacent downstream. The outgroup from *Frankia* sp. Eullc is in purple.

**Sequence alignments.** The degree of similarity across clade 2 is much greater than that of clade 1. The sequence conservation across the clades is represented in Fig. 3, which shows an alignment for all family members that have been experimentally characterized, either in this study or in studies previously reported in the literature. Those residues with 100% conservation across the entire family have a black background, while those conserved in clade 1 have a green background and those in clade 2 have a red background. Note that some residues were conserved in all but one sequence of the clade. These do not appear highlighted in the alignment. There are also positions that are conserved in all of clade 1 and have a different conserved residue in clade 2. These residues, indicated by an asterisk with a blue background, may be residues significant in substrate specificity between clades 1 and 2. A comprehensive family alignment can be found in Fig. S2 in the supplemental material. Further defining the catalytic and substrate specificity roles of residues conserved between and within each of the clades will require additional structural information (for example, that obtained from an X-ray structure determination).

**Selection of enzymes for experimental investigation.** The three cyanuric acid hydrolases with an experimentally determined function are located in group 1 (from *Moorella thermoacetica* ATCC 39073) and group 2 (AtzD and TrzD). None of the group 3

proteins had been functionally characterized until this study. The only known functional protein in clade 2 was barbiturase from *Rhodococcus erythropolis* JCM 3132, which was placed into group 5. To expand the knowledge of the functional sequence space in the family, five of the newly identified proteins were selected for further study, as described in the next section. The proteins were chosen either to represent a group with no functionally characterized members or as atypical proteins from groups containing characterized proteins.

The *Bradyrhizobium japonicum* USDA 110 protein was chosen due to its unusual placement in the tree compared to other *Bradyrhizobium* and *Rhizobium* strains. The *Methylobacterium* sp. 4-46 protein was one of 3 proteins chosen to represent group 3. The *Rhizobium leguminosarum* bv. *viciae* 3841 protein was chosen as another group 3 member because it was plasmid encoded. *Azorhizobium caulinodans* ORS 571 was found to have two family member proteins. The protein encoded by locus AZC\_3892 was placed into group 3, while the protein encoded by locus AZC\_3203 was the only potential family member, apart from the outgroup (the sequence from *Frankia* sp. Eullc), that was not placed into clade 1 or 2 (Fig. 2). Both *Azorhizobium* proteins were selected for further characterization to determine if they had the same or different functionalities. No clade 2 proteins were chosen

TABLE 1 Cyanuric acid hydrolase/barbiturase family proteins identified in this study and identities to enzymes of known function

Clade and organism <sup>a</sup>	GI no.	Locus name	% Identity to:			
			AtzD	TrzD	<i>Moorella</i> cyanuric acid hydrolase	Barbiturase
1						
<i>Clostridium asparagiforme</i> DSM 15981	225388419	CLOSTASPAR_02155	51	57	65	45
<b><i>Moorella thermoacetica</i> ATCC 39073</b>	83590946	Moth_2120	58	63	100	46
<i>Paenibacillus</i> sp. JDR-2	251798893	Pjdr2_4927	50	55	58	43
<i>Bacillus cellulosilyticus</i> DSM 2522	317127947	Bcell_1233	37	42	41	33
<i>Acidovorax citrullii</i> strain 12227	3659839		58	100	63	45
<i>Enterobacter cloacae</i> strain 99	13022197		58	100	63	45
<i>Aminobacter aminovorans</i> (formerly <i>Comamonas</i> sp. A2)	14029763	Partial sequence	58	100	63	45
<b><i>Bradyrhizobium japonicum</i> USDA 110</b>	27382392	blr7281	59	55	59	42
<b><i>Pseudomonas</i> sp. ADP</b>	32455884		100	58	58	43
<i>Pseudomonas nitroreducens</i>	285310188		100	58	58	43
<i>Comamonas</i> sp. A2	254972448		100	58	58	43
<i>Arthrobacter</i> sp. MCMB-436	47059636		100	58	58	43
<i>Arthrobacter</i> AD25	117583156		99.7	58	58	43
<i>Micromonas</i> sp. strain RCC299	255079800	MICPUN_59838	45	41	42	32
<i>Acidithiobacillus ferrooxidans</i> ATCC 53993 and ATCC 23270	198283056 218519570	Lferr_0922 AFE_0779	42	46	46	34
<i>Methylobacterium radiotolerans</i> JCM 2831	170751160	Mrad2831_4776	51	55	51	42
<i>Oceanicola granulosus</i> HTCC2516	89068460	OG2516_12979	44	44	44	37
<i>Agrobacterium vitis</i> S4	222106665	Avi_5653	48	50	56	43
<b><i>Rhizobium leguminosarum</i> bv. viciae 3841</b>	116254793	pRL100353	48	52	54	43
<i>Rhizobium leguminosarum</i> bv. trifolii WSM1325	241554234	Rleg_6452	48	52	53	44
<b><i>Methylobacterium</i> sp. 4-46</b>	170741972	M446_3816	46	47	47	37
<i>Bradyrhizobium</i> sp. strain BTai1	148252873	BBta_1315	47	47	52	38
<i>Bradyrhizobium</i> sp. strain ORS278	146343122	BRADO6319	47	48	54	40
<b><i>Azorhizobium caulinodans</i> ORS 571</b>	158425516	AZC_3892	50	52	55	41
2						
<i>Nocardioides</i> JS614	119718489	Noca_4270	43	44	45	72
<i>Thermobispora bispora</i> DSM 43833	296270184	Tbis_2213	43	49	50	77
<i>Streptosporangium roseum</i> DSM 43021	271967801	Sros_6541	42	46	48	74
<i>Geodermatophilus obscurus</i> DSM 43160	284988804	Gobs_0181	42	46	45	80
<i>Catenulispora acidiphila</i> DSM 44928	256391076	Caci_1879	45	47	47	81
<i>Nakamurella multipartita</i> DSM 44233	258655427	Namu_5328	43	45	46	84
<i>Nocardioides</i> sp. strain JS614	119715741	Noca_1505	43	46	46	86
<i>Intrasporangium calvum</i> DSM 43043	315588294	Intca_1068	43	47	48	80
<i>Janibacter</i> sp. strain HTCC2649	84497968	JNB_17813	42	45	45	85
<b><i>Rhodococcus erythropolis</i> JCM 3132</b>	46395595		43	45	46	100
<i>Rhodococcus erythropolis</i> SK121	229318388	RHOER0001_3381	43	45	46	99.7
<i>Rhodococcus erythropolis</i> PR4	226309369	RER_58820	43	45	46	99.7
<i>Rhodococcus opacus</i> B4	226362213	ROP_27990	42	46	45	92
<i>Rhodococcus jostii</i> RHA1	111022263	RHA1_ro05296	45	46	45	93
<i>Saccharomonospora viridis</i> DSM 43017	257056105	Svir_20970	44	46	47	85
<b><i>Azorhizobium caulinodans</i> ORS 571</b>	158424827	AZC_3203	49	48	52	43
<i>Frankia</i> sp. Eu11c	280963003	FraEu11c_3137	36	36	40	38

<sup>a</sup> Organisms containing homologs are in the same order as in the phylogenetic tree in Fig. 2. Organisms in which proteins were experimentally assayed are highlighted in bold.

for further characterization due to the limited diversity among the sequences. The genes for each of these AtzD/barbiturase family homologs were PCR amplified, cloned, and expressed in *E. coli*, and the resulting proteins were purified and assayed.

**Enzymatic activity and kinetic determinations.** The clustering of the *Bradyrhizobium japonicum* USDA 110 protein with the AtzD protein from *Pseudomonas* sp. ADP (12) suggested that this enzyme was a cyanuric acid hydrolase. However, with less than 59% identity to any of the experimentally confirmed cyanuric acid hydrolases, this functionality was ambiguous. Experimental stud-

ies of the recombinant protein confirmed it to be a cyanuric acid hydrolase. Furthermore, the proteins from group 3 of the phylogenetic tree, *Methylobacterium* sp. 4-46, *Rhizobium leguminosarum* bv. viciae 3841, and *Azorhizobium caulinodans* ORS 571 locus AZC\_3892, were also confirmed to have cyanuric acid hydrolase activity. These are the first functional assignments to this section of the tree, and they show that the cyanuric acid hydrolase enzymes are distributed throughout clade 1. In addition, the *Bradyrhizobium japonicum* USDA 110 and *Azorhizobium caulinodans* ORS 571 locus AZC\_3892 proteins were shown not to be reactive

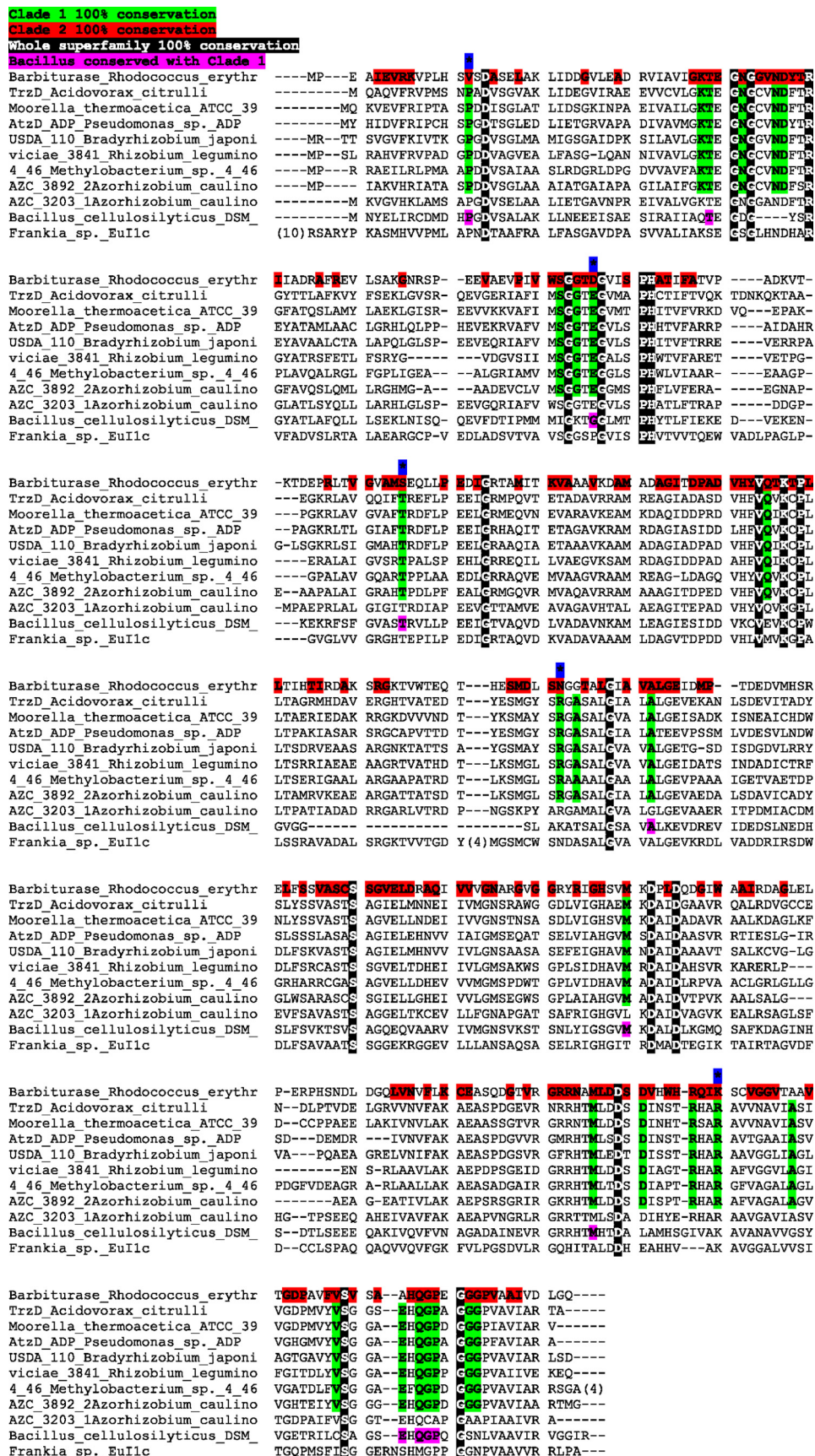


FIG 3 Sequence alignment of experimentally characterized and highly divergent cyanuric acid hydrolase/barbiturase family members. A black background indicates residues with 100% conservation over the entire family, a green background indicates 100% conservation across clade 1 (including sequences not in the current alignment), and a red background indicates 100% conservation across clade 2 (Note that only one sequence from clade 2 is shown in this alignment.) The blue background with an asterisk indicates different residues in clade 1 and clade 2 but 100% conservation within the respective clades.

TABLE 2 Kinetic constants of various cyanuric acid hydrolase/barbiturase family enzymes, using cyanuric acid as a substrate

Enzyme name and /or organism (reference)	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1} \text{M}^{-1}$ )
TrzD (20)	250	50	$5 \times 10^6$
TrzD (this study)	$14.2 \pm 0.4$	$58 \pm 7$	$(2.5 \pm 0.4) \times 10^5$
<i>Pseudomonas</i> sp. ADP AtzD (12)	$6.8 \pm 0.7$	$57 \pm 10$	$(1.2 \pm 0.3) \times 10^5$
<i>Pseudomonas</i> sp. ADP AtzD (this study)	$73 \pm 6$	$23 \pm 7$	$(3.2 \pm 1.2) \times 10^6$
<i>Moorella thermoacetica</i> ATCC 39073 (23)	10.6	110	$1.0 \times 10^5$
<i>Bradyrhizobium japonicum</i> USDA 110	$9.3 \pm 0.7$	$50 \pm 10$	$(1.9 \pm 0.5) \times 10^5$
<i>Rhizobium leguminosarum</i> bv. viciae 3841	$5 \pm 1$	$130 \pm 60$	$(3.8 \pm 2.5) \times 10^4$
<i>Methylobacterium</i> sp. 4-46	$17 \pm 2$	$69 \pm 16$	$(2.5 \pm 0.9) \times 10^5$
<i>Azorhizobium caulinodans</i> ORS 571 locus AZC_3892	$50 \pm 9$	$370 \pm 90$	$(1.3 \pm 0.6) \times 10^5$
<i>Azorhizobium caulinodans</i> ORS 571 locus AZC_3203	— <sup>a</sup>	—	—

<sup>a</sup> —, no activity with any substrate tested.

with barbituric acid. The second homolog from *Azorhizobium caulinodans* ORS 571, locus AZC\_3203, failed to display either cyanuric acid hydrolase or barbiturase activity, consistent with its position outside both clades 1 and 2 (Fig. 2). The AZC\_3203 protein was further tested for activity with other potential substrates, including uracil, thymine, 6-azauracil, 5-azacytosine, cytosine, 2,4,5-trihydroxypyrimidine, 5-nitrobarbuturic acid, creatinine, 5,6-dihydroxyuracil, hydantoin, ammelide, ammeline, allantoin, alloxan, urea, L-asparagine, and biuret. No hydrolysis of any of these compounds could be detected by spectroscopy or HPLC.

Steady-state kinetic constants were determined to investigate whether those parameters would indicate that cyanuric acid hydrolysis was a major physiological function for these proteins. In general, physiological enzymes show  $k_{\text{cat}}/K_m$  ratios of at least  $10^4 \text{ M}^{-1} \text{ s}^{-1}$  for their preferred substrates. For comparative purposes, AtzD from *Pseudomonas* sp. ADP and TrzD from *Acidovorax citrullii* strain 12227 were also purified and kinetic values determined. Note that the AtzD protein has been demonstrated to be part of an operon that is regulated partly by cyanuric acid (13) and that TrzD was isolated from a bacterium that grows on cyanuric acid as a sole nitrogen source.

Stability studies with the *Pseudomonas* sp. ADP enzyme found that when the enzyme was frozen, a substantial loss in activity occurred. However, storage of the enzyme at 4°C resulted in stable activity for more than 1 month. For this reason, all enzymes were stored under these new conditions and activity monitored throughout the study to ensure no detectable loss of activity. The  $k_{\text{cat}}$  for the *Pseudomonas* sp. ADP enzyme, stored in this way, was 10-fold higher than that previously published (11). However, purified TrzD had a  $k_{\text{cat}}$  that was 10-fold lower than that previously published (20). The  $k_{\text{cat}}$  and  $K_m$  values for the newly cloned cyanuric acid hydrolases were each within an order of magnitude of each other (Table 2). The  $k_{\text{cat}}$  values ranged from 5 to  $73 \text{ s}^{-1}$  and the  $K_m$  values from 23 to 370  $\mu\text{M}$ . These give  $k_{\text{cat}}/K_m$  values that ranged from  $4 \times 10^4$  to  $5 \times 10^6$ . These values suggested that cyanuric acid hydrolysis is the physiological function of these enzymes. In contrast, the published values for the barbiturase are lower for  $k_{\text{cat}}$  and higher for  $K_m$ . Thus, the  $k_{\text{cat}}/K_m$  is lower ( $K_m = 1 \text{ mM}$ ,  $k_{\text{cat}} = 1.6 \text{ s}^{-1}$ , and  $k_{\text{cat}}/K_m = 1.6 \times 10^3$ ) (34). However, the genome and physiological context indicate that barbituric acid hydrolysis is the physiological function for this enzyme.

**Demonstration that cyanuric acid hydrolases and barbiturases are isofunctional with their respective substrates.** Based on overall sequence relatedness and multiple-sequence alignments, it seemed odd that barbiturases and cyanuric acid hydrolases would

catalyze different reactions. Biuret had been reported as the product of the cyanuric acid hydrolase reaction (8, 12, 20, 23), including in one previous study using  $^{13}\text{C}$  NMR (36). However, in that previous  $^{13}\text{C}$  NMR experiment, the reaction mixture was analyzed at a pH of 4.0, a pH at which carboxybiuret, if formed, would be highly unstable. In that context, this experiment was reexamined here.

In the present study,  $^{13}\text{C}$  NMR was used to analyze reaction mixtures poised at pH 7.7. Cyanuric acid is a symmetrical molecule (Fig. 1A), and the three carbon atoms appeared as a single resonance (157.7 ppm) in the  $^{13}\text{C}$  NMR spectrum, as expected (Fig. 1A and 4A). Biuret is also symmetrical (Fig. 1A), and carbonate/bicarbonate contains a single carbon atom; therefore, single resonances were expected. Experiments confirmed this, with chemical shifts of 157.5 and 160.4 ppm, respectively. The three carbon atoms in the proposed carboxybiuret intermediate would be bonded to different substituents (Fig. 1A) and would be expected to appear as three separate resonances. Prior to enzyme addition, a stable signal for [U- $^{13}\text{C}$ ]cyanuric acid was observed at 157.7 ppm (Fig. 4A). The addition of cyanuric acid hydrolase from *Moorella thermoacetica* ATCC 39073 led to a decrease in the cyanuric acid resonance of approximately 40% and, concomitantly, showed three new signals (157.1 ppm, 156.8 ppm, and 155.0 ppm), which we attributed to the presence of carboxybiuret (Fig. 4B). No bicarbonate/carbonate signal was observed at this time. As the reaction proceeded (Fig. 4C), a peak attributed to bicarbonate/carbonate appeared at 160.4 ppm. The resonance(s) at 157.5 to 155.7 ppm became broad and misshapen due to the appearance of biuret carbon atoms resonating at 157.5 ppm and overlapping with the residual cyanuric acid peak at 157.7 ppm. As the reaction continued, the cyanuric acid peak shifted slightly upfield, likely due to increased acidity of the reaction mixture. The biuret peak increased and sharpened (157.5 ppm) (Fig. 4D). Concurrently, the bicarbonate/carbonate peak also increased. At this stage of the reaction, the integration of the bicarbonate/carbonate-biuret signals yielded a molar stoichiometry of 1:1, as expected. Also, the ratio of the proposed carboxybiuret carbon resonances was 1:1:1, as expected. (Fig. 4D). Within 2 h, only bicarbonate/carbonate and biuret were detected (Fig. 4E). Formation of carboxybiuret via  $^{13}\text{C}$  NMR was observed with the cyanuric acid hydrolases from *Pseudomonas* sp. ADP, *Moorella thermoacetica* ATCC 39073, and *Azorhizobium caulinodans* ORS 571 (locus AZC\_3892). These results suggested that cyanuric acid hydrolases uniformly catalyze a hydrolytic amide bond cleavage that is analogous to the barbiturase reaction (Fig. 1).

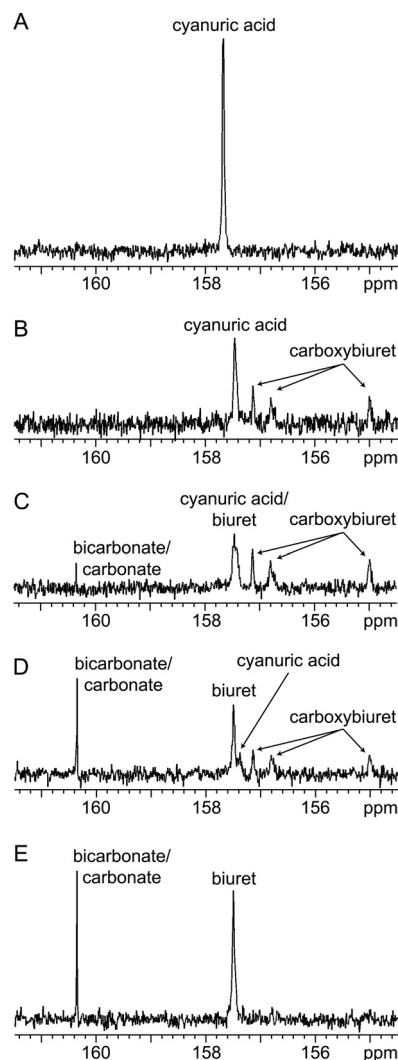


FIG 4  $^{13}\text{C}$  NMR of cyanuric acid hydrolase biotransforming  $[\text{U}-^{13}\text{C}]$  cyanuric acid to  $[\text{U}-^{13}\text{C}]$  carboxybiuret, followed by nonenzymatic decarboxylation. (A)  $[\text{U}-^{13}\text{C}]$  cyanuric acid without enzyme; (B to E)  $[\text{U}-^{13}\text{C}]$  cyanuric acid with enzyme at 9 min (B), 14 min (C), 41 min (D), and 112 min (E).

Attempts to synthesize carboxybiuret were unsuccessful, and, to our knowledge, its synthesis has not been described in the literature. In light of this, we also employed mass spectrometry with electrospray ionization to support the identification of carboxybiuret. Cyanuric acid with natural isotopic abundance was incubated with cyanuric acid hydrolase and observed over time. A parallel experiment was performed using  $[\text{U}-^{13}\text{C}]$  cyanuric acid. In negative-ion mode, we observed mass ions consistent with the formation, and subsequent decomposition, of carboxybiuret adducts over the time course. Two pairs of ions were examined in detail. Each pair contained one ion from the experiment with unlabeled cyanuric acid and a corresponding heavier ion from the experiment with  $[\text{U}-^{13}\text{C}]$  cyanuric acid. In the case of the ion pair with the nominal masses of 244 and 247 Da, these were attributed to carboxybiuret combining with potassium acetate (see Fig. S3 in the supplemental material). In the case of the ion pair with masses of 347 and 352 Da, these could be generated from carboxybiuret in complexation with biuret and potassium acetate or phosphoric

acid. We did not observe the parent ion of carboxybiuret. This is likely because it was too unstable to survive the electrospray or the mass spectrometer analyzer and thus was seen only when complexed with another ion that provided some protection. In total, the mass spectrometry data supported the conclusion that carboxybiuret is the intermediate generated by cyanuric acid hydrolase.

**Evidence against the AtzD/barbiturase family linkage to the amidohydrolase superfamily.** Initially, the barbiturase from *Rhodococcus erythropolis* JCM 3132 was identified as a member of the amidohydrolase superfamily due to the presence of a C-terminal HxH site in the protein sequence and a 1:1 zinc-to-subunit ratio (34). The homologous AtzD protein, however, lacked any observable bound metal (12). The current study further establishes that, beyond these two proteins being homologous, the reactions that they catalyze are completely analogous. Having homologous proteins catalyzing analogous reactions with two different mechanisms, one involving a metal and one without, is highly unusual, prompting additional investigation into this matter.

Extensive sequence comparisons were conducted here to assess AtzD/barbiturase family linkages or their absence. Results from pblast, PSI-BLAST, Shotgun, and HMMER were unable to link any AtzD/barbiturase family member to an outside family or superfamily, including the amidohydrolase superfamily. Likewise, analysis of over 33,000 amidohydrolase sequences found in the Structure Function Linkage Database (29) failed to connect to any member of the AtzD/barbiturase family.

The amidohydrolase proteins comprise an extensive superfamily, usually containing one or two metals that are liganded by a set of conserved histidines and, in many cases, carboxylic acid residues. The amino acid ligands span the length of the linear protein sequence, with the HxH site being the first, located in the N-terminal part of the protein. The protein folds into a  $(\beta/\alpha)_8$  barrel in which the metal ligands, located in conserved positions within the secondary structure, converge into the center of the barrel to coordinate the metal (33). Secondary-structure predictions for the AtzD/barbiturase family were not able to establish similarity in structure or to find metal ligands comparable to those in the amidohydrolase superfamily. The only commonality between this superfamily and the barbiturase is the HxH site. The presence of an HxH site alone is not enough to link proteins to the amidohydrolase superfamily, especially since the amidohydrolase site is in the N terminus and the barbiturase site is in the C terminus of the protein. Countless other proteins also have an HxH site without being related to the amidohydrolase superfamily (6, 17, 19, 27, 38). Furthermore, analysis of the AtzD/barbiturase family sequences reveals that this HxH site, initially identified in a single protein from *Rhodococcus erythropolis* JCM 3132, is not completely conserved (Fig. 3). If this motif were required for metal binding and catalysis, conservation across the entire protein family would be expected.

Previous studies on enzymes from *Acidovorax citrulli* strain NRRLB-12227, *Pseudomonas* sp. ADP, and *Moorella thermoacetica* ATCC 39073 found no stimulation from adding divalent metal ions (12, 20, 23). However, in the present study, we did analyze for the presence or absence of a stoichiometrically bound metal. The cyanuric acid hydrolases from *Pseudomonas* sp. ADP, *Moorella thermoacetica* ATCC 39073, and *Bradyrhizobium japonicum* USDA 110 and the barbiturase from *Rhodococcus erythropolis* JCM 3132 were subjected to metal analysis under the same conditions.



Zinc, iron, cobalt, nickel, copper, manganese, magnesium, lead, cadmium, and chromium were looked for. Only zinc and nickel were above background levels, and the amounts of those metals combined accounted for less than 0.1 metal per subunit. This differs greatly from the case for amidohydrolase superfamily members, which usually have 1 to 2 metals per subunit, as determined in our laboratory by this same method. These data suggested that both the cyanuric acid hydrolases and barbiturase are not metalloproteins.

## DISCUSSION

This study is the first to describe the cyanuric acid hydrolases and barbiturases as a protein family. Previously, the barbiturases were suggested to be zinc enzymes in the amidohydrolase superfamily, but sequence analysis and metal determination conducted here argue against that family assignment and place them, along with the cyanuric acid hydrolases, in a unique protein family. This study further established that both enzymes catalyze common chemistry: a hydrolytic ring amide bond cleavage reaction, resulting in the opening of a heterocyclic 6-membered ring. The NMR and mass spectrometry experiments conducted here show for the first time that cyanuric acid hydrolases release carboxybiuret and not biuret.

The known set of proteins in this protein family was expanded from 4 to 41. Still, this is a rare family, appearing on an average of once in every 200 sequenced genomes (from a set of 6,423 genomes). The lack of a crystal structure within this set prevents the identification of the fold. Fold recognition/homology modeling programs such as SUPERFAMILY (15), pGenTHREADER (25), pDomTHREADER (25), Phyre2 (21), and SWISS-MODEL (1) were unable to provide any insights into structural classification. However, sequence comparisons, followed up by purification and activity measurements, allowed us to differentiate barbiturases from cyanuric acid hydrolases. The latter are the larger set of proteins known at this time, mainly due to their prevalence in *s*-triazine-degrading bacteria. The mobility of *s*-triazine degradation genes and high sequence identity of this group (group 2 in Fig. 2) suggest that these proteins may be part of a resurgence of cyanuric acid hydrolases due to the large amount of anthropomorphic *s*-triazine chemicals being produced currently. *s*-Triazine herbicides and melamine alone are produced in quantities greater than one hundred million pounds and one billion pounds, respectively (22).

Cyanuric acid is found naturally, but its occurrence derives from spontaneous rather than metabolic chemistry. Cyanuric acid has been recovered from meteorites (16), and *s*-triazines have been identified in “primordial soup” experiments (18). Cyanuric acid also is formed intracellularly during the radiative decay of purine nucleotides. These observations are consistent with the data in this paper that suggest an ancient set of genes without a strong selection pressure for their prevalence.

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