Molecular Probing and Phylogenetic Analysis of Glutamate synthase of Arthrobacter nicotianae.

W Mazhawidza, M Somuah, N Rajendran

Citation

W Mazhawidza, M Somuah, N Rajendran. *Molecular Probing and Phylogenetic Analysis of Glutamate synthase of Arthrobacter nicotianae.*. The Internet Journal of Microbiology. 2008 Volume 7 Number 2.

Abstract

Introduction: Glutamate synthase is an oxidoreduactase transaminating enzyme that catalyzes the conversion of L-glutamate to L-glutamine with the aid of cofactors like NADP, NAD, FMN, Fe and sulfur. Its activities are in part to regulate the levels of glutamate and glutamine in a manner dependent on energy (ATP) and metabolite, ammonia. Methods and results: In this study we were able to demonstrate the presence of glutamate synthase by employing PCR utilizing genomic DNA isolated from soil bacteria Arthrobacter nicotianae PR. Cloning, sequencing and alignment revealed glutamate synthase with conserved amino acid residues specific to this group of functional proteins. 3D analysis of the GltS using Cn3D 4.1 software revealed well conserved alpha and beta sheets that in part form the TIM barrel and cofactor bonding site. Amino acids that bind the phosphate group of the cofactor were revealed. Un-rooted phylogenetic tree revealed unique distant metabolic relationship, potential development and adaptation of Artherobacter nicotianae PR compared to other bacteria including strains in the Athrobacter group. Conclusion: The phylogenetic divergence of GltS in the matching bacteria suggests possible evolutionary pressure being exerted by the local environment among other factors

INTRODUCTION

Glutamate synthase (GltS) is an enzyme complex of NADP, iron, sulfur and FMN, that catalyses the reversible oxidoreductive biosynthesis of L-glutamate from 2-oxoglutarate and L-glutamine using ammonia (1). This reaction occurs in bacterial, yeast and as well as plant cells. This enzymatic complex interaction plays a central in amino acid metabolism as well as maintaining control of ammonia levels (1). Other names for the enzyme are glutamine-ketoglutaric aminotransferase and glutamine amide-2-oxoglutarate aminotransferase. The mechanism of action involves NADP or FMN as a cofactor. The enzyme can also utilize other cofactors like FAD, iron and sulfur as alternative cofactors. It is on the bases of these cofactors that Glt(S) is classified into three groups, the NADPH-dependent in bacteria, ferredoxin-dependent in plants and NAD (P)H-dependent in other lower animals. Specifically, this group of enzymes acts on CH-NH2 as donor groups and the cofactors as the acceptors.

Glutamate is very central to the metabolism of amino acids as its carbon chain or amide group can be directed into different biosynthetic pathways. Whilst the mechanism of L-glutamine biosynthesis in lower organisms and plants is mediated by glutamate synthase, it is part of a dual pathway that is dependent on the abundance energy in the form of ATP, the levels of ammonia and glutamine which is convertible back to glutamate(2). Specifically E. coli has a dual pathway whose activities are inhibited or activated by ATP and ammonia (3, 4). These alternate pathways allow the bacteria to regulate the levels of glutamate, glutamine and ultimately assimilate ammonia. The same enzyme mechanisms are also used by other bacterium like cyanobacterium to regulate ammonia levels(5). This type of dual mechanism to regulating the biosynthesis of amino acids is thought to be utilized by other organisms like algae, yeast and fungi(6).

Previous cross species phylogenetic analysis revealed unique phenomena about glutamate synthase and homologous enzymes in prokaryotes (7). Lateral gene transfer, was suggested by the scattered ness of glutamate synthase and homologous genes (7).

Given the critical role this enzyme plays in amino acid metabolism it is important to understand its evolutionary position across any related species particularly the large community of soil inhabiting bacteria. In our study, we

attempted to perform phylogenetic analysis of glutamate synthase in soil isolated Arthrobacter nicotianae PR with matching results from NCBI blast search of microbe protein databases. The identity of Arthrobacter nicotianae was established based on chemical analysis as well as 16S rRNA and other genes (Accugenix, DE). To reveal the presence of glutamate in Arthrobacter nicotianae, PCR was performed employing previously published primers (8). PCR products were resolved in a 1% agarose gel, cloned and sequenced as described in materials and methods below. The newly cloned sequence and the matches from NCBI linked databases were analyzed using Lasergene suite software (DNAstar), and allowed us to reveal a unique evolutionary relationship between Arthrobacter nicotianae and a very diverse group of microbes.

MATERIALS AND METHODS

BACTERIAL CULTURE METHODS AND DNA ISOLATION:

Nutrient Broth culture (10mL) of Arthrobacter nicotianae strain PR was grown at 30 °C, by picking a single colony from a nutrient agar stock plate prepared earlier by streak-plate technique. Genomic DNA was isolated using DNA bactozol kit (Molecular Research Center, OH, USA). The genomic DNA isolation was confirmed by resolving the bacterial lysate in agarose gel before use in PCR.

PCR CONDITIONS:

CER1 and EMT1 primers (8) were used in this study. Primers were synthesized at Integrated DNA Technologies, Coralville, IA, USA. The PCR reactions were carried out as standardized earlier(9). Thermocycler (Eppendorf Mastercycler personal, Eppendorf) was used to cycle the PCR. The following conditions: 95 ºC (5 min), 95 ºC (1 min), 55 ºC (1 min), and 72 ºC (3 min) for 30 cycles were adopted.

SUBCLONING AND DNA SEQUENCING:

Amplicons obtained from PCR were sub cloned in E. coli, using manufacturer's protocol instructions (Invitrogen USA). Positive clones were screened using IPTG/X-Gal (Fermentas, USA) with Ampicillin (Sigma, USA). Positive colonies were grown in ampicillin /LB liquid culture followed by plasmid isolation using Pure Yield Plasmid Miniprep System (Promega, USA). The clones were confirmed by EcoR1 restriction before sequencing using a T3 and or T7 primer at the Center for Genetics and Molecular Medicine (CGeMM) DNA Core facility of the University of Louisville, KY, USA. Further analysis of the DNA sequence was done using NCBI-blast and MegAlign software (lasergene, DNAstar).

SEQUENCE ANALYSIS, CONSTRUCTION OF PHYLOGENETIC TREE AND 3D ANALYSIS:

After confirmation of the DNA sequence using NCBI vector contamination software (vecscreen), further computational analysis was conducted. Homologous nucleotide and or amino acid sequence search was performed using NCBI blast search of protein databases (http://www.ncbi.nlm.nih.gov/BLAST/). The newly determined Glutamate Synthase sequence was deposited in GenBank and an accession number FJ979920 was obtained using the BankIT:GenBank (www.ncbi.nlm.nih.gov/BankIT/) submission program. To confirm the specificity signature sequence conserved amino acid residue search was performed during multiple sequence alignment. MegAlign software was then used to generate the phylogenetic tree.

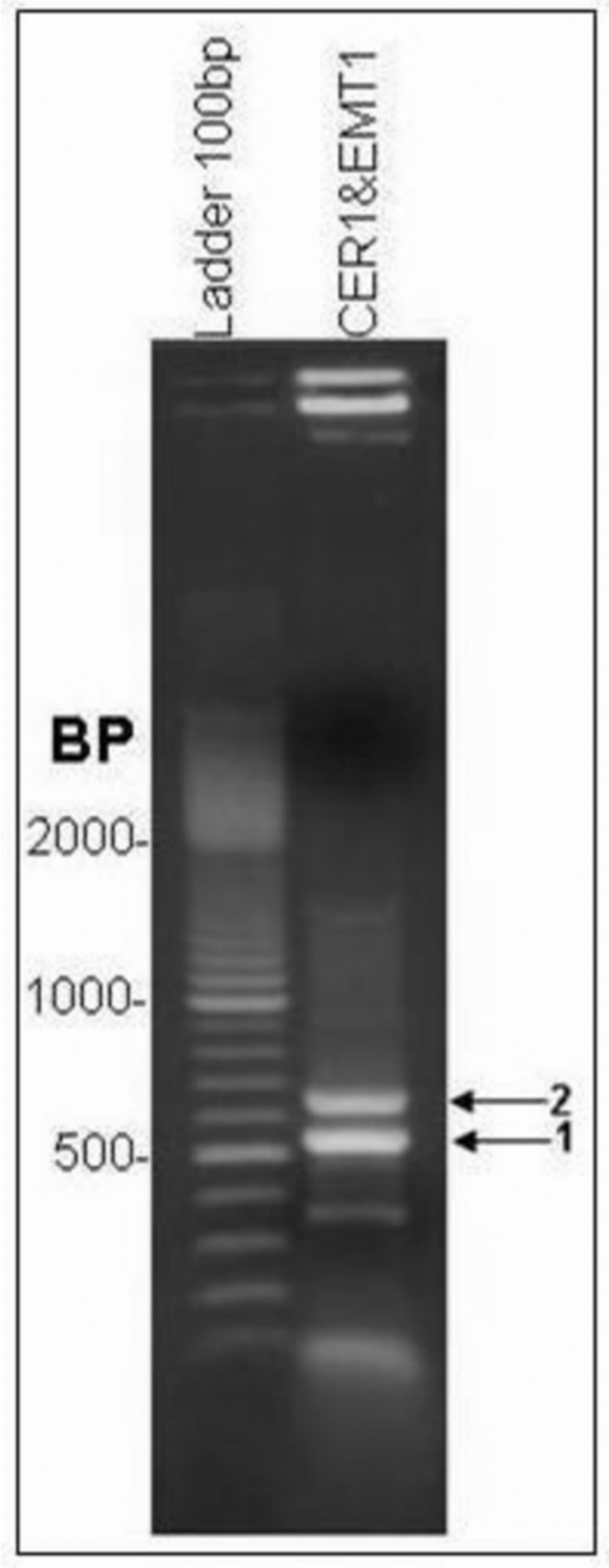
To determine the domain structure of the clone, Cn3D 4.1 (NCBI) software was used. The newly cloned sequence was compared to 3D domain model constructed from multiple sequence alignment of many peptides in the same class. Domain alignment was performed to demonstrate common and consensus regions. Images were exported from Cn3D software as JPEG.

RESULTS

Nucleotide primers (8) designed from conserved domains of nonribosomal peptide synthetase were used in PCR to amplify corresponding regions on Arthrobacter nicotianae genomic DNA. As shown in Figure 1, we were able to amplify a ~550 bp and a ~650 bp sections of DNA from Arthrobacter nicotianae using the protocol described in materials and methods. The smaller amplicon was of no interest for this report. The submission of a trimmed 516 bp sequence to the Genebank has been completed and the accession number FJ979920 assigned. NCBI blast searching of microbe protein databases revealed a match of the newly cloned sequence to glutamate synthase from a diverse group of bacteria.

Figure 1

Fig 1. Electrophoresis of PCR products. Primers were employed against the genomic DNA of the newly isolated soil bacteria under conditions described in materials and methods. PCR generated two amplicons. The larger amplicon was of interest to this report.



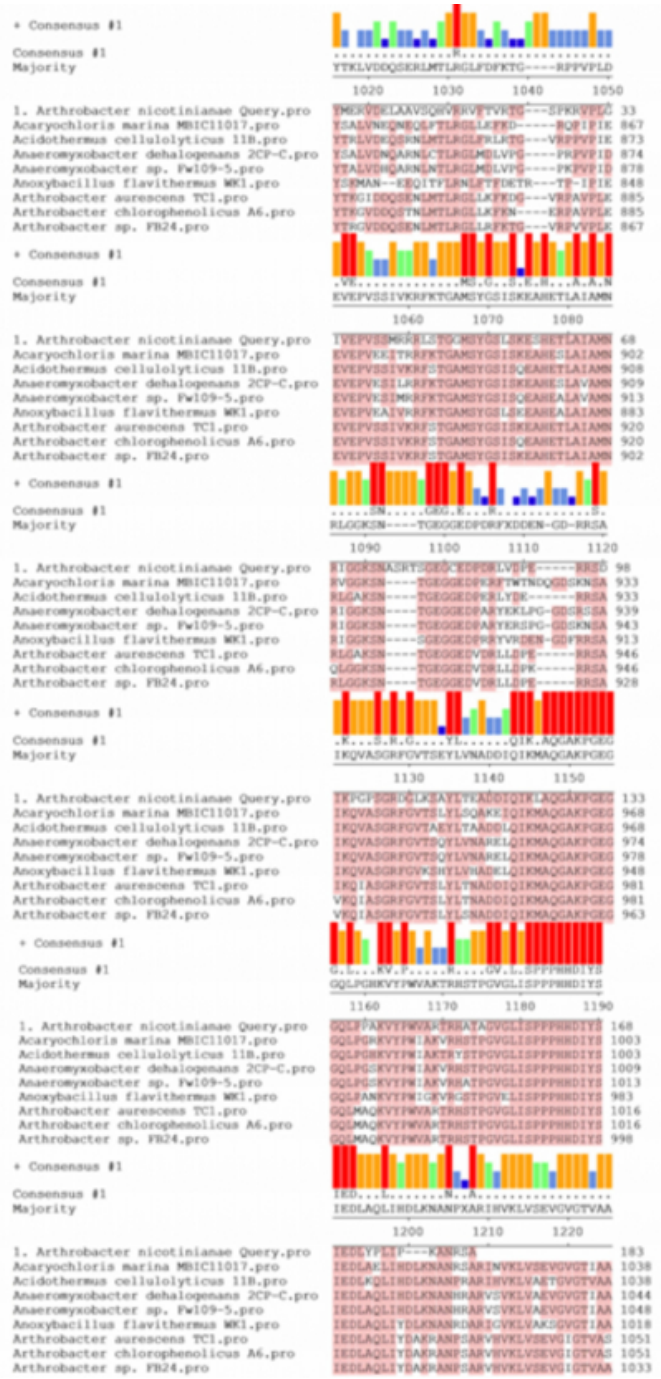
SEEKING THE CONSENSUS SEQUENCES,

HOMOLOGOUS AMINO ACID RESIDUES AND 3D DOMAINS

In an effort to find a putative identity of the gene partial sequence, we analyzed the amplicon sequence by multiple sequence alignment using multiple sequence alignment software, MegAlign, DNAstar (Fig 2). Alignment was performed with potential matches obtained from NCBI blast search of microbe protein databases. Using ClustalW, we revealed the presents of conserved amino acid residues shown in figure 2. A thorough search of the conserved domains revealed that the newly cloned sequence matched in part to the TIM barrel of the GltS as well as having FMN binding consensus amino acid residues (Fig. 2). A comparison of the TIM barrel primary structure and our Arthrobacter nicotianae GltS sequence showed amino acids considered critical in binding FMN, a cofactor required for the function of the enzyme (10-12). These amino acids found in the new GltS sequence are residues; GGMSYGS- position 1165 to 1171, GEG- position 1058 to 1060, and K- position 1145. We noted a unique substitution of a conserved Q by P in position 1123. As shown in figure 3, there are other conserved amino acids revealed by this analysis that probably do not play a role in FMN binding. It is our opinion based on the structure of GltS that these residues may have a role in forming and maintaining the TIM barrel tertiary structure.

Figure 2

Fig 2. Multiple sequence alignment of Glutamate synthase reveals consensus amino acid residues. The homologous matches obtained from BLASTX search were aligned using Megalign program (Lasergene, DNAstar). Shown are only 8 of the 72 bacteria strains revealed by the NCBI blast search of microbe protein databases. ClustalW shows the presents of conserved amino acids residues in specific positions indicated



Tertiary structure analysis of the newly sequenced GltS was performed using Cn3D 4.1 software (NCBI) (13). Conserved amino acid residues of the structural alpha and beta sheets

that form the TIM barrel are present in Arthrobacter nicotianae (Fig. 3). As shown in figure 3, on the left are three different views of the domains that form the TIM barrel (11) and on the right are the TIM barrel domains in the newly cloned GltS sequence. Included in the images is the putative binding site for the phosphate group of the cofactor i.e. FMN (Fig. 3).

PHYLOGENETIC POSITIONING

Phylogenetic tree of partial glutamate synthase sequence cloned from arthrobacter nicotianae along with other microbial sequences and genomes was performed using MegAlign software (Fig. 4). Analysis included all 72 matches from the NCBI blast search (supplementary 1 data not shown). As shown in Figure 4, similar strains of bacteria clustered together. Notable is that Arthrobacter nicotianae did not cluster together with other Arthrobacter despite having common consensus amino acid residues. This suggests possible diversity and divergent evolutionary development in the bacterial species.

Figure 3

Fig 3. 3D analysis reveals conserved TIM barrel domain in GltS. The tertiary structure of the newly cloned GltS was compared with that of similar multiple sequences in protein databases. This was performed using Cn3D 4.1 software. The 3D model comparison revealed common conserved alpha and beta sheets that partly form the TIM barrel and the binding site of cofactor phosphate group. On the right are three views of the TIM barrel from multiple sequence alignment of sequences on NCBI link protein databases and in the left are three similar images showing domains in common with the newly cloned GltS sequence.

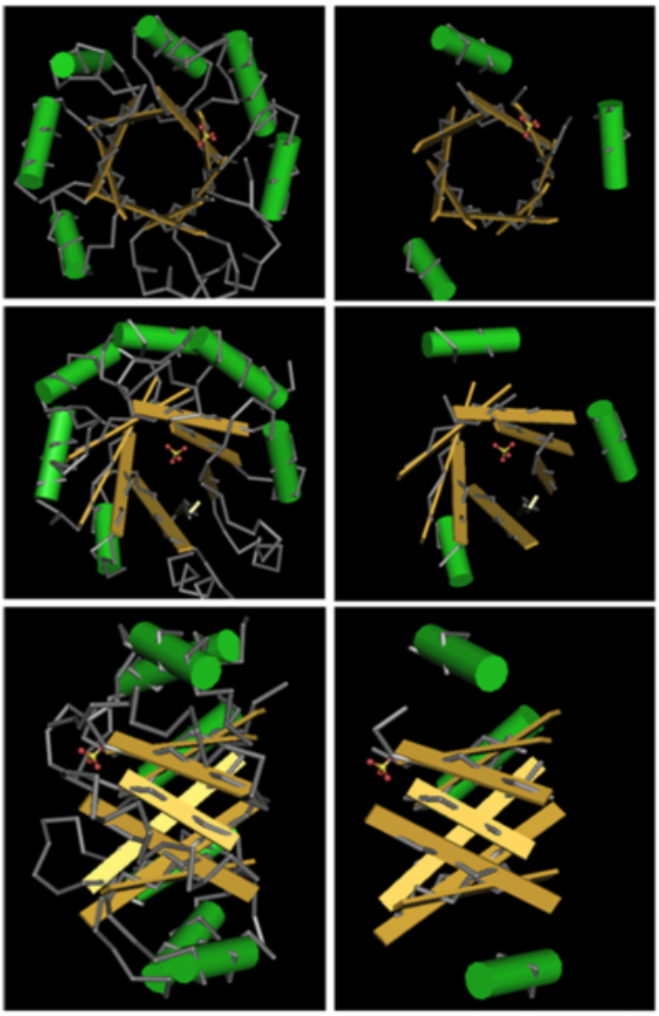
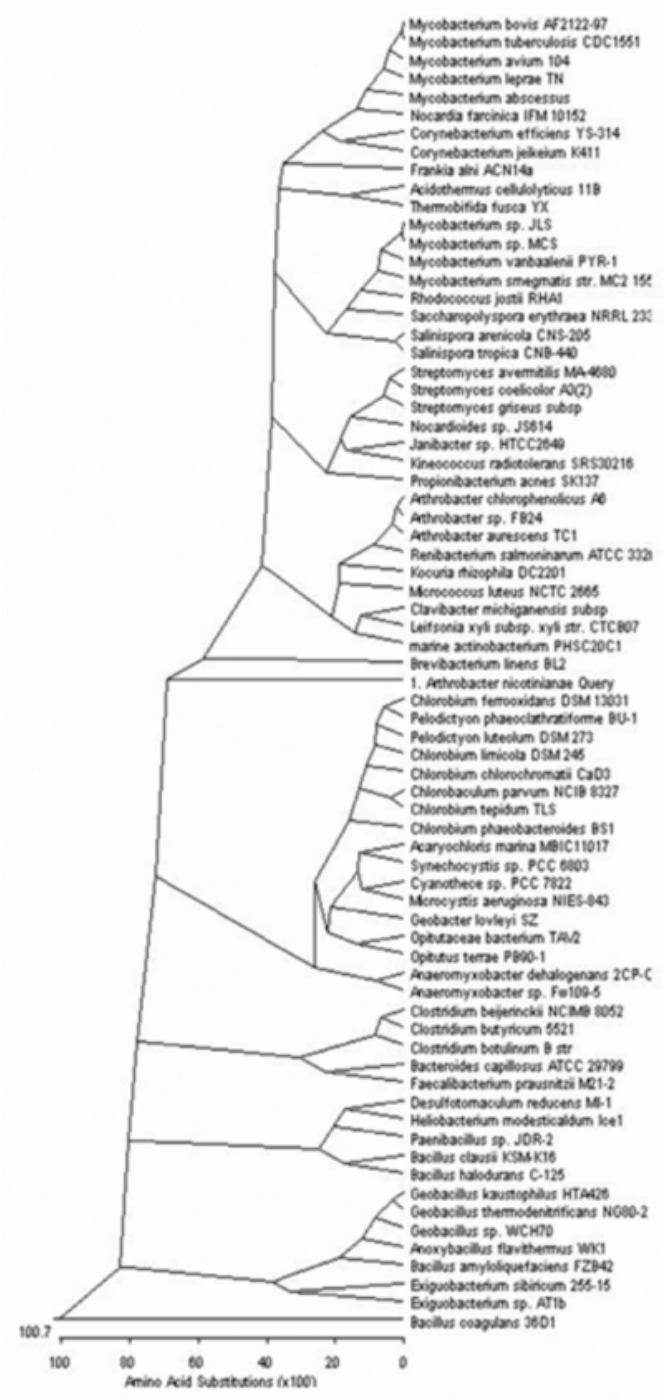


Figure 4

Fig 4. The phylogenetic tree constructed and based on published microbe Glutamate Synthase. Sequences of and other related microbes aligned in Fig. 3 and Supplementary 1 were compared and used in phylogenetic tree construction using MegAlign software. The tree is unrooted and each node and length of arm represents sequence divergence estimated as number of amino acid substitution as the tree branches.



DISCUSSION

In our study we employed a PCR approach in revealing part of the genome of a newly isolated and identified soil bacterium. As noted before Arthrobacter nicotianae was identified by chemical approach as well as sequencing the ribosomal genes (data not shown). The sequencing and analysis of the cloned segment of Athrobacter nicotianae genome revealed the putative identity of the sequence as part of the glutamate synthase enzyme. Specifically the sequence matched to the TIM barrel that is a tunnel-like tertiary structure formed by alpha and beta sheets as shown in figure 3. The TIM barrel is central to the function of many enzymes including GltS. The newly cloned sequence revealed amino acid residues that form the alpha and beta sheets as well as consensus residues that form the cofactor (FMN) binding site.

GltS is a large protein with many functional domains(14). What we accomplished is cloning the middle section of the gene with structural function as well as the binding of cofactors. As shown in figure 3, the GltS cloned has amino acid residues critical for FMN binding. Missing from the clone are amino acid residues that form the substrate binding site as well as ferrodoxin cluster (14). To our knowledge this is the first time GltS has been cloned in A. nicotianae and will significantly contribute to the current databases as well as understanding of the GltS and bacterial evolutionary development.

GLTS AND PHYLOGENETIC ANALYSIS

Glutamate synthase is phylogenetically interesting as well as metabolically pivotal to the biology of bacteria and other organisms (15-20). Previous phylogenetic analysis revealed that GltS had undergone lateral gene transfer from bacteria to a common ancestor of animals, fungi, and plants (21). Our analysis demonstrates a close relationship between strains of the same type of bacteria as expected. Unique is that Arthrobacter nicotianae as well as Mycobacterium sp did not seem to cluster with other Arthrobacter or Mycobacterium bacteria. This suggests possible divergence in the phylogeny of Glutamate synthase in these bacteria. The expression of genes, such as GltS are dependent on environmental factors (22). The growth of Arthrobacter bacteria was previously shown to be in part dependent on the levels of ammonia and type of carbon source like methylamine (22). If this is true, then the evolutionary development of the Arthrobacter bacteria may be tied to the local environment, but that is beyond the scope of this study.

It is important to highlight a few bacteria that matched with the newly cloned GltS. In mycobacterium, the activity of

GltS and Glutamine synthase have been shown to be dependent on nitrogen source and a levels of ammonia in the environment (23-25). Nocardia sp and Frankia similarly demonstrates enhanced expression of GS/GOGAT pathway as the major pathway for the assimilation of ammonia with glutamate as the source of nitrogen (26-28). GltS harbored in Arthrobacter nicotianae also shows similarity to those of soil inhabiting exiguobacteria suggesting influence from a possible common environmental factor.

The availability and use of software like Cn3D 4.1 has potential to positively impact analysis and discovery of life science data like we have not seen before (13, 29-33). By importing the newly cloned GltS sequence into the software we were able to align the primary structure as well as determine conserved domains and view them in their putative three dimensional forms. This approach to analysis of genomic data as we have seen has the potential to identify and rapidly unveil the metabolism as well as the phylogentic position of the organism.

CONCLUSION

We revealed the presence of glutamate synthase in Arthrobacter nicotianae PR and its phylogenetic relationship to other microbes. Multiple sequence alignment of all matches revealed well conserved amino acids residues that form the TIM barrel and the FMN binding site. Though the phylogenetic analysis did not show close relationship with other Arthrobacter strains, a search for alignment of functional domains demonstrated the presence of alpha and beta sheets that form the TIM barrel structure as well as cofactor binding site. The revelation of alignment of domains of the newly cloned sequence in 3D analysis confirmed putative identity and suggested the mechanism of the enzyme to be similar to that of TIM binding superfamily of enzymes. The molecular and phylogenetic analysis of glutamate synthase in Arthrobacter nicotianae helped to shade insight into diversity of the enzyme in strains of the same bacteria potentially due to local environs. Glutamate is a significant enzyme in the metabolism of amino acids and assimilation if ammonia and its pylogenetic revealation in Arthrobacter nicotianae and other bacteria are critical to understanding evolutionary diversity in amino acid metabolism.

ACKNOWLEDGEMENTS

This work was supported by National Science Foundation, USA (Grant # MCB-0639356 to Narayanan Rajendran). Authors thank ASM/JGI(DOE) and Drs. Brad Goodner, Cheryl Kerfeld, Kathleen Scott and Sam Donovan of ASM Spring-2009 Bioinformatics Institute for sequence alignment, phylogenetic and bioinformatics training.

References

1. Binda C, Bossi RT, Wakatsuki S, Arzt S, Coda A, Curti B, et al. Cross-talk and ammonia channeling between active centers in the unexpected domain arrangement of glutamate synthase. Structure2000 Dec 15;8(12):1299-308.
2. Forde BG, Lea PJ. Glutamate in plants: metabolism, regulation, and signalling. J Exp Bot2007;58(9):2339-58.
3. Helling RB. Why does Escherichia coli have two primary pathways for synthesis of glutamate? J Bacteriol1994 Aug;176(15):4664-8.
4. Helling RB. Pathway choice in glutamate synthesis in Escherichia coli. J Bacteriol1998 Sep;180(17):4571-5.
5. Chavez S, Lucena JM, Reyes JC, Florencio FJ, Candau P. The presence of glutamate dehydrogenase is a selective advantage for the Cyanobacterium synechocystis sp. strain PCC 6803 under nonexponential growth conditions. J Bacteriol1999 Feb;181(3):808-13.
6. Huth J, Liebs P. [Nitrogen regulation in microorganisms]. Zentralbl Mikrobiol1988;143(3):179-94.
7. Anderson CM, Bridges RJ, Chamberlin AR, Shimamoto K, Yasuda-Kamatani Y, Swanson RA. Differing effects of substrate and non-substrate transport inhibitors on glutamate uptake reversal. J Neurochem2001 Dec;79(6):1207-16.
8. Horwood PF, Burgess GW, Oakey HJ. Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in Bacillus cereus. FEMS Microbiol Lett2004 Jul 15;236(2):319-24.
9. Rajendran N, Rajnarayanan RV, Demuth DR. Molecular phylogenetic analysis of tryptophanyl-tRNA synthetase of Actinobacillus actinomycetemcomitans. Z Naturforsch [C]2008 May-Jun;63(5-6):418-28.
10. Marchler-Bauer A, Bryant SH. CD-Search: protein domain annotations on the fly. Nucleic Acids Res2004 Jul 1;32(Web Server issue):W327-31.
11. Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, et al. CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res2007 Jan;35(Database issue):D237-40.
12. Marchler-Bauer A, Anderson JB, Cherukuri PF, DeWeese-Scott C, Geer LY, Gwadz M, et al. CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res2005 Jan 1;33(Database issue):D192-6.
13. Chakrabarti S, Lanczycki CJ, Panchenko AR, Przytycka TM, Thiessen PA, Bryant SH. Refining multiple sequence alignments with conserved core regions. Nucleic Acids Res2006;34(9):2598-606.
14. NALBANTOGLU B. Analysis of Functional Domains on Glutamate Synthase. Turk j biology2000. p. 197-213.
15. Vanoni MA, Curti B. Structure--function studies on the iron-sulfur flavoenzyme glutamate synthase: an unexpectedly complex self-regulated enzyme. Arch Biochem Biophys2005 Jan 1;433(1):193-211.
16. van den Heuvel RH, Svergun DI, Petoukhov MV, Coda A, Curti B, Ravasio S, et al. The active conformation of glutamate synthase and its binding to ferredoxin. J Mol Biol2003 Jun 27;330(1):113-28.
17. van den Heuvel RH, Ferrari D, Bossi RT, Ravasio S, Curti B, Vanoni MA, et al. Structural studies on the synchronization of catalytic centers in glutamate synthase. J Biol Chem2002 Jul 5;277(27):24579-83.
18. Petoukhov MV, Svergun DI, Konarev PV, Ravasio S,

van den Heuvel RH, Curti B, et al. Quaternary structure of Azospirillum brasilense NADPH-dependent glutamate synthase in solution as revealed by synchrotron radiation x-ray scattering. J Biol Chem2003 Aug 8;278(32):29933-9.
19. Navarro F, Martin-Figueroa E, Candau P, Florencio FJ. Ferredoxin-dependent iron-sulfur flavoprotein glutamate synthase (GlsF) from the Cyanobacterium synechocystis sp. PCC 6803: expression and assembly in Escherichia coli. Arch Biochem Biophys2000 Jul 15;379(2):267-76.
20. Morandi P, Valzasina B, Colombo C, Curti B, Vanoni MA. Glutamate synthase: identification of the NADPH-binding site by site-directed mutagenesis. Biochemistry2000 Feb 1;39(4):727-35.
21. Andersson JO, Roger AJ. Evolutionary analyses of the small subunit of glutamate synthase: gene order conservation, gene fusions, and prokaryote-to-eukaryote lateral gene transfers. Eukaryot Cell2002 Apr;1(2):304-10.
22. De Boer L, Brouwer JW, Van Hassel CW, Levering PR, Dijkhuizen L. Nitrogen metabolism in the facultative methylotroph Arthrobacter P1 grown with various amines or ammonia as nitrogen sources. Antonie Van Leeuwenhoek1989 Oct;56(3):221-32.
23. McCarthy CM, Alvarez ME. Influence of nitrogen source and growth status on glutamine synthetase and glutamate synthase activity in Mycobacterium avium. Can J Microbiol1985 Mar;31(3):211-3.
24. McCarthy CM. Continuous culture of Mycobacterium avium limited for ammonia. Am Rev Respir Dis1983 Feb;127(2):193-7.
25. Singh P, Venkitasubramanian TA. Ammonia assimilation by Mycobacterium smegmatis during growth on different nitrogenous sources. Indian J Biochem Biophys1978 Apr;15(2):139-41.
26. Palaniappan C, Gunasekaran M. Ammonium assimilation in Nocardia asteroides. Mycopathologia1993 Nov;124(2):69-72.
27. Mei BG, Jiao RS. Purification and properties of glutamate synthase from Nocardia mediterranei. J Bacteriol1988 Apr;170(4):1940-4.
28. Schultz NA, Benson DR. Enzymes of ammonia assimilation in hyphae and vesicles of Frankia sp. strain CpI1. J Bacteriol1990 Mar;172(3):1380-4.
29. Porter SG, Day J, McCarty RE, Shearn A, Shingles R, Fletcher L, et al. Exploring DNA structure with Cn3D. CBE Life Sci Educ2007 Spring;6(1):65-73.
30. Willis RC, Hogue CW. Searching, viewing, and visualizing data in the Biomolecular Interaction Network Database (BIND). Curr Protoc Bioinformatics2006 Jan;Chapter 8:Unit 8 9.
31. Marchler-Bauer A, Panchenko AR, Shoemaker BA, Thiessen PA, Geer LY, Bryant SH. CDD: a database of conserved domain alignments with links to domain three-dimensional structure. Nucleic Acids Res2002 Jan 1;30(1):281-3.
32. Wang Y, Bryant S, Tatusov R, Tatusova T. Links from genome proteins to known 3-D structures. Genome Res2000 Oct;10(10):1643-7.
33. Hogue CW. Cn3D: a new generation of three-dimensional molecular structure viewer. Trends Biochem Sci1997 Aug;22(8):314-6.

Author Information

Williard Mazhawidza, PhD
Biology, Kentucky State University

Michael Somuah
Biology, Kentucky State University

Narayanan Rajendran, PhD
Biology, Kentucky State University