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Original Article

In Silico Analysis of Glutaminase from Different Species of *Escherichia* and *Bacillus*

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What's Known

- Glutaminase is one of the important enzymes in food and pharmaceutical industries. In recent years, glutaminase has been identifie from various biological sources such as bacteria, fungi, yeasts, and mammals. But *Escherichia* and *Bacillus* spp. are the major producers of glutaminase.

What's New

- *Escherichia* and *Bacillus* spp. are the main producers of glutaminase. We performed a comprehensive *in silico* study of bacterial glutaminase producers, especially various *Bacillus* and *Escherichia* strains, regarding their physicochemical properties and phylogenetic relations in order to fin new enzyme sources.

Abstract

Background: Glutaminase (EC 3.5.1.2) catalyzes the hydrolytic degradation of L-glutamine to L-glutamic acid and has been introduced for cancer therapy in recent years. The present study was an *in silico* analysis of glutaminase to further elucidate its structure and physicochemical properties.

Methods: Forty glutaminase protein sequences from different species of *Escherichia* and *Bacillus* obtained from the UniProt Protein Database were characterized for homology search, physiochemical properties, phylogenetic tree construction, motif, superfamily search, and multiple sequence alignment.

Results: The sequence level homology was obtained among different groups of glutaminase enzymes, which belonged to superfamily serine-dependent β-lactamases and penicillin-binding proteins. The phylogenetic tree constructed indicated 2 main clusters for the glutaminases. The distribution of common β-lactamase motifs was also observed; however, various non-common motifs were also observed.

Conclusion: Our results showed that the existence of a conserved motif with a signature amino-acid sequence of β-lactamases could be considered for the genetic engineering of glutaminases in view of their potential application in cancer therapy. Nonetheless, further research is needed to improve the stability of glutaminases and decrease their immunogenicity in both medical and food industrial applications.

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Keywords ● *Escherichia* ● *Bacillus* ● Glutaminase ● Computer simulation

Introduction

Glutaminase or glutamine amidohydrolase (EC 3.5.1.2) catalyzes the hydrolytic deamination of L-glutamine, leading to the generation of L-glutamate and ammonium.[1,2] In recent years, glutaminase has attracted much attention given its proposed applications in both food and pharmaceuticals industries.

Glutaminase has been recognized in bacteria, fungi, yeasts, and mammals.[2-4] It plays an essential role in nitrogen metabolism, involving glutaminolysis. While mitochondrial glutaminase is elevated in some tumor types and is frequently upregulated in MYC-transformed cells,[5] it is thought to be a potential chemotherapeutic target.[6] Moreover, *Achromobacter* glutaminase exerted antileukemic influences in patients with acute myeloid leukemia or acute lymphoblastic leukemia in a preliminary clinical trial.[7] Glutaminase–asparaginase obtained from *Pseudomonas* 7A

showed considerable antitumor activity in some studied mice,[7] particularly when applied together with glutamine antimetabolites. Glutaminase has provided hope as an encouraging therapeutic agent for the healing of diseases caused by retroviruses. It has also attracted significant attention from the pharmaceutical industry on the strength of its potential applications as an anticancer agent. In this regard, there are 2 genes for the glutaminase available in *Escherichia coli*, namely *yneH* (308 aa) and *ybaS* (310 aa). The *ybaS* gene encodes an enzyme that is only active in acidic pH but not in physiological pH. On the other hand, the *yneH* gene has optimum activity in physiological pH and is suitable for cancer therapy purposes.

Alongside its demonstrated potential as an antileukemic agent, glutaminase is generally regarded as a key enzyme in controlling the taste of fermented foods such as soy sauce, especially in Asian countries.[8,9] Most of the essential flavor components of fermented condiments are amino acids generated by the enzymatic hydrolysis of proteins contained in raw food materials; and among them, L-glutamic acid is a broadly recognized flavor-enhancing amino acid.[8] Moreover, L-glutamate (monosodium glutamate) is a prominent umami taste factor. Hence, the deamination of glutamine is an important route in the food industry with the aim of enhancing the umami taste. For instance, the distinctive taste of fermented soy sauce is ascribed chiefly to glutamic acid (concentrations of 0.7 to 0.8% per total nitrogen).[2] The activity of glutaminase, accountable for the fabrication of glutamic acid, renders it a chief supplement for the period of soy-sauce fermentation. Efforts to enhance the glutamate content of soy sauce by means of salt and thermotolerant glutaminases have attracted much attention.[10]

Among glutaminase producers, *Escherichia* and *Bacillus* spp. are well studied microorganisms in all aspects, especially some *Bacillus* spp. have been given GRAS (generally regarded as safe) status by the Food and Drug Administration (FDA). Furthermore, therapeutic enzymes such as asparaginase have been obtained from *Escherichia* spp.[11] Asparaginase also has relative glutaminase activity, which makes it a good candidate for therapeutic purposes.

In the context of increased practical applications for glutaminase, we performed an *in silico* analysis of 40 glutaminase protein sequences from *Escherichia* and *Bacillus* spp. To our knowledge, this is the first research to analyze glutaminase protein sequences using bioinformatics approaches. Drawing upon a variety of bioinformatics tools, we sought to characterize glutaminase protein sequences in terms of biochemical traits, multiple sequence alignment (MSA), homology search, motif, phylogenetic tree construction, and superfamily allocation.

Materials and Methods

The amino-acid sequences of glutaminase from various *Escherichia* and *Bacillus* spp. were obtained from the UniProt Protein Database and the Expert Protein Analysis System (ExPASy) proteomics server. Physiochemical data were provided through ProtParam via the ExPASy server (the proteomics server of the Swiss Institute of Bioinformatics). The Fast Adaptive Shrinkage/Thresholding Algorithm (FASTA) format of the sequences was utilized for subsequent analyses. Various tools in the proteomics server (ProtParam, ClustalW, Compute pI/Mw, Protein Calculator, and ProtScale)[12] were implemented to calculate/deduce different physiochemical features of the glutaminases from the protein sequences. The molecular weights (kDa) of the various glutaminases were computed by adding the mean isotopic mass of the amino acid in the enzyme and deducting the mean isotopic mass of 1 water molecule. The pI of the enzyme was computed using the pKa value of the amino acid according to Bjellqvist et al.[13] (1993). The atomic compositions of the glutaminases were obtained using ProtParam, available at ExPASy. The aliphatic index values of the various glutaminase protein sequences were determined using ProtParam (ExPASy).[12] The grand average of hydropathicity (GRAVY) and the instability index were estimated using the Kyte and Doolittle[12] and Guruprasad[14] methods, respectively. CLC Sequence Viewer 7 was used[15] for dendrogram construction via the neighbor-joining method (NJ).[16] For domain search, Pfam (http://sanger.ac.uk/software/Pfam/search.html) and InterPro (http://www.ebi.ac.uk/interpro) were used. Motif analysis was done using MEME (http://meme.sdsc.edu/meme/meme.html) and MOTIF search (http://www.genome.jp/tools/motif). The protein conserved motifs deduced by MEME were subjected to biological functional analysis using protein BLAST, and the motifs were studied using InterProScan to find the best possible match based on the highest similarity score. Forty glutaminase protein sequences with accession numbers showing different species of *Escherichia* and *Bacillus* are listed in table 1.

Results

Forty glutaminase protein sequences from different species of *Escherichia* and *Bacillus*

Table 1: Biochemical features of glutaminase protein sequences from different species of *Escherichia* and *Bacillus*

S. no	Accession number	Source organisms	Number Of amino acids	Molecular weight	Theoretical pI	Total number of negatively charged residues (Asp+Glu)	Total number of positively charged pesidues (Arg+Lys)	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)
1	P0A6W0	*E. coli* (strain K12)	308	33515.5	5.98	29	25	43.79	98.80	0.133
2	P0A6W2	*E. coli* O157:H7	308	33515.5	5.98	29	25	43.79	98.80	0.133
3	P0A6W1	*E. coli* O6:H1	308	33515.5	5.98	29	25	43.79	98.80	0.133
4	C3T9T2	*E. coli*	308	33515.5	5.98	29	25	43.79	98.80	0.133
5	B1LF91	*E. coli* (strain SMS-3-5)	308	33515.5	5.98	29	25	43.79	98.80	0.133
6	B7N4U9	*E. coli* O17:K52:H18	308	33514.6	6.17	28	25	44.06	98.80	0.133
7	D3QT73	*E. coli* O55:H7	308	33515.5	5.98	29	25	43.79	98.80	0.133
8	D3GSG1	*E. coli* O44:H18	308	33515.5	5.98	29	25	43.79	98.80	0.133
9	B7URQ1	*E. coli* O127:H6	308	33515.5	5.98	29	25	43.79	98.80	0.133
10	C8TRP0	*E. coli* O26:H11	308	33515.5	5.98	29	25	43.79	98.80	0.133
11	B7L7M4	*E. coli* (strain 55989)	308	33515.5	5.98	29	25	43.79	98.80	0.133
12	E3PM25	*E. coli* O78:H11	308	33515.5	5.98	29	25	43.79	98.80	0.133
13	B7LRC7	*E. fergusonii* (ATCC 35469)	308	33532.6	6.25	29	26	38.87	96.30	0.120
14	Q1RBP3	*E. coli* (strain UTI89)	308	33515.5	5.98	29	25	43.79	98.80	0.133
15	C8U8J5	*E. coli* O103:H2	308	33515.5	5.98	29	25	43.79	98.80	0.133
16	B6IAS7	*E. coli* (strain SE11)	308	33515.5	5.98	29	25	43.79	98.80	0.133
17	A7ZLY0	*E. coli* O139:H28	308	33515.5	5.98	29	25	43.79	98.80	0.133
18	C8UPW4	*E. coli* O111:H	308	33515.5	5.98	29	25	43.79	98.80	0.133
19	B7MMY8	*E. coli* O45:K1	308	33515.5	5.98	29	25	43.79	98.80	0.133
20	D8BUE3	*E. coli* MS 196-1	308	33515.5	5.98	29	25	43.79	98.80	0.133
21	W0B1N6	*E. albertii* KF1	308	33542.6	6.24	28	25	41.24	97.86	0.146
22	A0A0F6C473	*E. coli* Xuzhou21	308	33515.5	5.98	29	25	43.79	98.80	0.133
23	K4WSD4	*E. coli* O111:H8	308	33515.5	5.98	29	25	43.79	98.80	0.133
24	W8ZS57	*E. coli* ST131	308	33515.5	5.98	29	25	43.79	98.80	0.133
25	A0A0E0V4K9	*E. coli* O7:K1	308	33515.5	5.98	29	25	43.79	98.80	0.133
26	S1HXQ0	*E. coli* KTE108	308	33515.5	5.98	29	25	43.79	98.80	0.133
27	S1HI46	*E. coli* KTE103	308	33515.5	5.98	29	25	43.79	98.80	0.133
28	K5CT98	*E. coli* AD30	308	33515.5	5.98	29	25	43.79	98.80	0.133
29	H5V2J6	*E. hermannii* NBRC 105704	308	33312.5	6.31	28	26	40.55	104.87	0.210

(*Contd*...)

Table 1: (*Continued*)

S. no	Accession number	Source organisms	Number of amino acids	Molecular weight	Theoretical pI	Total number of negatively charged residues (Asp+Glu)	Total number of positively charged residues (Arg+Lys)	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)
30	A0A090V5E8	*E. vulneris* NBRC 102420	308	33760.6	5.85	32	26	52.44	95.03	0.020
31	B1ELZ3	*E. albertii* (strain TW07627)	308	33489.6	6.07	28	24	41.24	97.86	0.169
32	O07637	*B. subtilis* (strain 168)	309	34012.3	5.78	36	33	23.17	94.01	0.026
33	A8FCU0	*B. pumilus* (strain SAFR-032)	309	33600.8	5.90	35	32	33.86	87.18	-0.011
34	A7Z4A6	*B. amyloliquefaciens* subsp. Plantarum	309	33989.2	5.65	38	33	34.97	87.73	-0.046
35	G4NWR9	*B. subtilis* subsp. spizizenii	309	34001.4	5.77	36	33	26.55	94.66	0.016
36	D4FW74	*B. subtilis* subsp. natto	309	34040.4	6.03	36	34	24.04	94.01	0.022
37	R9TT78	*B. licheniformis* 9945A	309	33601.7	5.03	39	29	17.37	92.49	0.047
38	A0A060LRN5	*B. lehensis* G1	309	33947.8	4.85	42	28	28.24	91.91	-0.079
39	I3E8E0	*B. methanolicus* MGA3	309	33751.4	8.31	32	35	29.15	96.28	0.088

retrieved from the UniProt Protein Database were characterized for homology search, MSA, biochemical features, phylogenetic tree construction, superfamily, and motif search using a variety of bioinformatics tools.

The biochemical features for these glutaminases are listed in table 1. The total number of amino-acid residues was 308 for the *Escherichia* spp. and 310 for the *Bacillus* spp., with variable molecular weights. The pI value ranged from 4.85 to 8.31. The variability was also observed among these glutaminases in terms of other physiochemical parameters such as positively charged amino-acid residues, negatively charged residues (Asp and Glu), and hydropathicity (GRAVY) (table 1). The sequence-based analysis of the aliphatic index among these glutaminases in the *Escherichia* spp. revealed homogeny with a range of ~98 with the exception of *E. hermannii*, which had a value of 104.87. As for the *Bacillus* spp., a variety of aliphatic indices were observed, from 87.18 to 96.28.

The MSA and homology search of these 40 glutaminase protein sequences disclosed a stretch of conserved regions (figure 1). However, a few highly conserved amino acids were also observed for many of the sequences (figure 1).

The phylogenetic tree constructed based on the glutaminase protein sequences using the NJ method revealed 2 major clusters for the *Escherichia* and *Bacillus* spp., denoting the sequence-level similarity of the glutaminase protein sequences (figure 2). Several *Escherichia* species-specific clusters for glutaminase, namely *E. fergusonii*, *E. albertii*, *E. hermannii*, and *E. vulneris*, were also observed (figure 2). A similar profile was achieved from the phylogenetic tree constructed using the unweighted pair group method with arithmetic mean (UPGMA) and the minimum-evolution method (data not shown).

These glutaminases, when subjected to the SUPERFAMILY tool on the ExPASy server,[17] revealed their identity: They belonged to superfamily serine-dependent β-lactamases and penicillin-binding proteins. The motif analysis of the glutaminases from the *Escherichia* and *Bacillus* spp. revealed the existence of more than 40 absolutely conserved residues including the predicted β-lactamase motif 1,[18] a catalytic diad Ser-X-X-Lys. Moreover, β-lactamase sequence motif 3 (Lys/Arg-Ser/Thr-Gly) was identifiable in the glutaminases (Lys259-Ser260- Gly261), while only Ser (Ser160) could be identified for the Ser-Asp-Asn triad of class A β-lactamase motif 2 (figure 1).

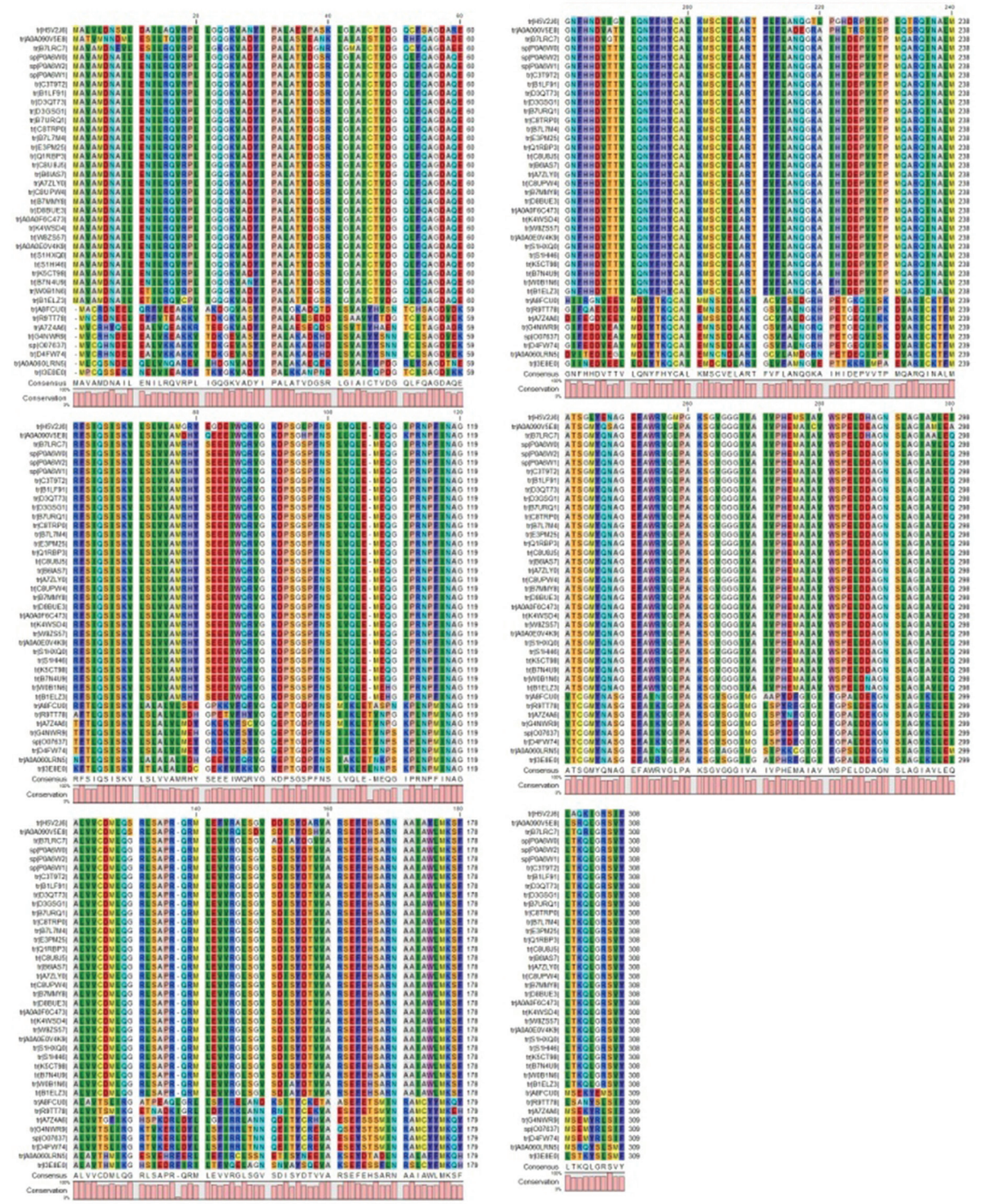

Figure 1: Multiple sequence alignment of glutaminase protein sequences shows maximum homology from amino-acid residues 60–120. The represented accession numbers of the bacteria and their complete details are provided in table 1.

In addition to the conserved β-lactamase sequences, varied motifs were also obtained. The MotD (flagellar motor protein) motifs (LETILRQVRPLIGQGKVADYIPALATVEG SRLGIAICTVDGQLFQAGDAQERFSIQSISKV) along with WisP family C-Terminal Region (RGLSGVSDIAYDTVVAR SEFEH SARNAAIA WLMKSFGNFHHD VTTVLQNYFHYC) were observed among the *Escherichia* spp., but not in the *Bacillus* spp. However, various motifs were observed in the *Bacillus* spp., including aminoacyl-tRNA ligase (QEPTGDPFNSIIKLETVN P S K P L N P M I N A G A L V V T S L I R G R T VKERLDYLLSFIRRLTN) motif in *B. subtilis* spp. (strain 168, spizizenii and natto) and TENA motif (IRILTFVQELAGNSN VAYSQE VAKSEFESS

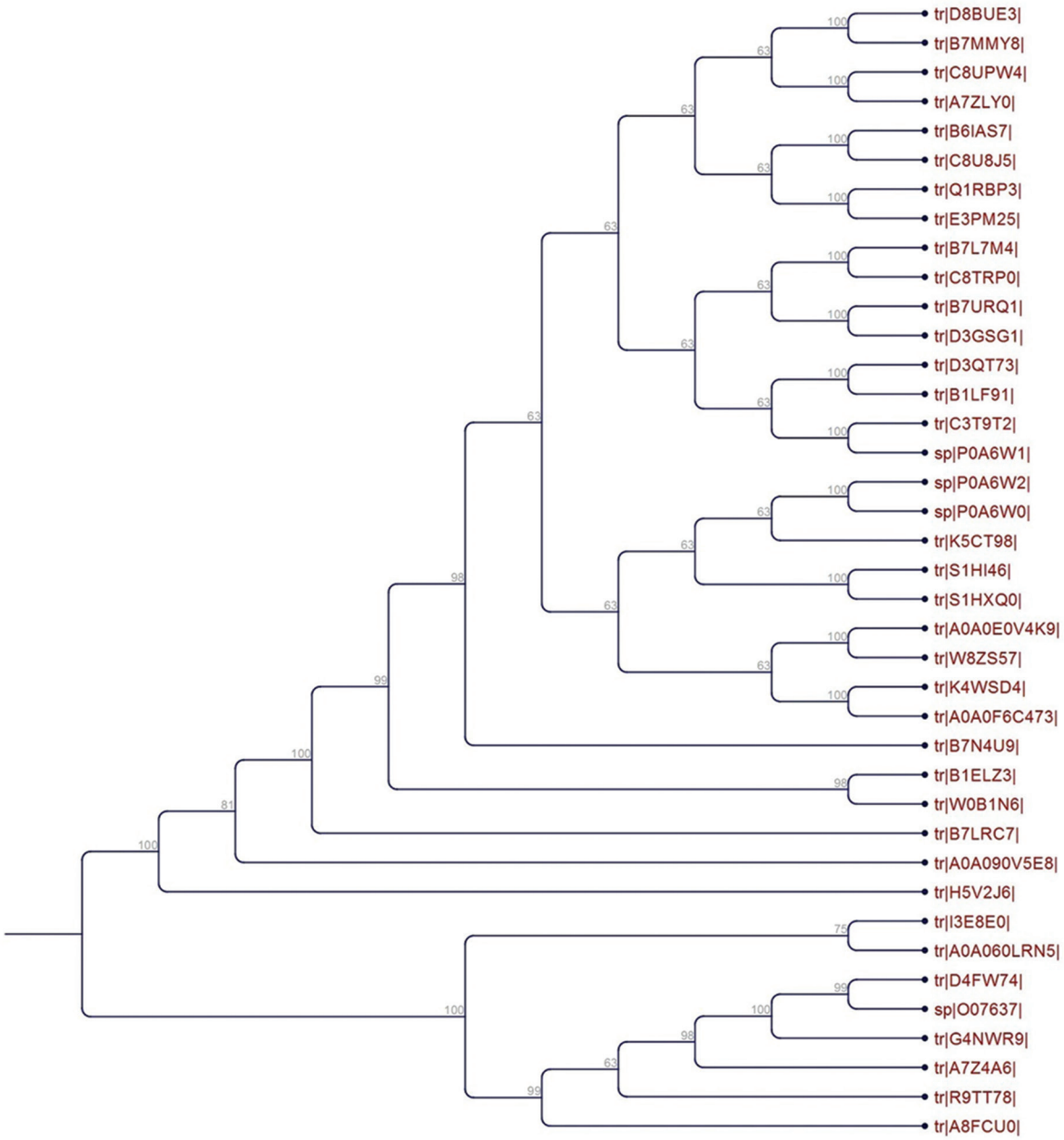

Figure 2: Phylogenetic tree constructed using the neighbor-joining (NJ) method based on the glutaminase protein sequences from different species of *Escherichia* and *Bacillus* is depicted here. The represented accession numbers of the bacteria and their comprehensive details are provided in table 1.

FLNRSLCY) in *B. methanolicus*, which may help to secret proteases such as glutaminase into the extracellular environment.

Discussion

Glutaminase (EC 3.5.1.2) catalyzes the hydrolytic deamination of L-glutamine to L-glutamic acid and has a vital task in cellular nitrogen metabolism. In mammals, both kidney and liver types of glutaminase are present. However, it is widely distributed in almost all organisms, including bacteria. In this regard, *Escherichia* and *Bacillus* spp. attract a great deal of attention due to their potential in medical and industrial applications. Nowadays, many bioinformatics tools are harnessed in different biological fields such as protein engineering and vaccinology to lower the costs and improve the accuracy of experimental investigations.[19-21]

In this research, we performed an *in silico* study of glutaminases from 2 bacteria, namely *Escherichia* and *Bacillus* spp. The biochemical traits for these glutaminase enzymes are depicted in table 1. The sequence-based analysis of the aliphatic index among these

glutaminases in the *Escherichia* spp. revealed homogeny with a range of ~98 with the exception of *E. hermannii*, which had a value of 104.87. As for the *Bacillus* spp., a variety of aliphatic indices were observed, from 87.18 to 96.28. The aliphatic index of a protein sequence is an extent of the relative volume occupied by aliphatic side chain of valine, alanine, isoleucine, and leucine amino acids. An increase in the aliphatic index is considered to represent an elevation in the thermostability of globular proteins.[15] The glutaminases of *Escherichia* and *Bacillus* spp. appear to be thermostable given the high value of their aliphatic index.[15] The instability index is considered for the measurement of the *in vivo* half-life of a protein.[14] It has been reported that proteins that possess an *in vivo* half-life >16 hours have an instability index <40, while those that possess an *in vivo* half-life <5 hours display an instability index >40.[22] The computed instability index of the glutaminases from the *Escherichia* spp. was found to be half-life <5 hours, with the exception of *E. fergusonii* (half-life >16 h). In contrast, all the *Bacillus* spp. represented an instability index <40, which showed a half-life >16 hours and indicated that they were good candidates for medical and industrial applications.

The MSA and homology search divulge several homologies. The presence of conserved small sequence patches with important roles in the authentication of protein and helix-coil transition has been previously stated.[23,24] Structural and sequence homology methods principally represent the global similarities between the compared glutaminases.[25] However, in general terms, the molecular role of a glutaminase is confined to its known active site, which may include in an interaction with the peptide linkage of proteins. Keeping the core structural constituent of the active site is necessary for maintaining the functional activity of the enzyme. Therefore, protein comparisons that focus on structural similarities in a global sequence may fail to spot proteins with conserved active sites but divergent structures and sequences.[26] The conserved region observed between these glutaminases could be utilized for designing degenerate primers for polymerase chain reaction (PCR)-based amplification and cloning of reputed glutaminase genes from the diverse species of *Escherichia* and *Bacillus*.

Our sequence analysis of the glutaminases using the SUPERFAMILY tool on the ExPASy server revealed that they belonged to superfamily serine-dependent β-lactamases and penicillin-binding proteins. Poorly characterized glutaminases belong to the huge cluster of serine penicillin-binding proteins and β-lactamases, which have a shared evolutionary origin and apportion the protein fold, catalytic mechanism, and structural motifs.[18] This huge set of enzymes comprises DD-peptidases, glutaminases, 3 classes of well-characterized serine β-lactamases (A, C, and D), and transpeptidases.[27] β-Lactamase (EC 3.5.2.6) catalyzes the hydrolysis of an amide bond (N–CO) in the β-lactam ring of the antibiotics of the penicillin/cephalosporin family contributing to the most common mechanism of bacterial resistance to β-lactam antibiotics, while penicillin-binding proteins encompass transpeptidase, carboxypeptidase, and transglycosylase activities and have a part in the biosynthesis of the bacterial cell wall.[18,28,29] The representatives of all DD-peptidase and β-lactamase families have been described together biochemically and structurally, and the molecular mechanisms of the catalytic activity have been recognized.[30-33] Motif analysis represents 2 major β-lactamase motifs, including class C β-lactamases. Class C β-lactamases include a conserved Tyr residue (Tyr150 in AmpC from *Enterobacter cloacae*) in place of Ser in motif 2,[18] which also has no apparent counterpart in glutaminase sequences. Consequently, motif analysis denotes that glutaminases keep motifs 1 and 3 of β-lactamases but vary in motif 2. These sequences could be exploited for the expression and diversity analysis of glutaminase enzymes and confer valuable data for a better understanding of the structure and function of glutaminase. To that end, further research is required to assess the immunogenicity and thermal tolerance of glutaminase and improve its stability in different environmental conditions.

Conclusion

Our *in silico* evaluation of glutaminase protein sequences from diverse species of *Escherichia* and *Bacillus* clearly disclosed a sequence level similarity which could be helpful in cloning putative genes using degenerate primers designed from the conserved sequences. The phylogenetic clustering, conserved motif sequences, and discrepancy between the biochemical traits of the different glutaminases in this study could be deemed critical information for investigating new glutaminases and comparing them with other types of β-lactamases for the further classification and application of diverse β-lactamases. The operational characterization of amino-acid residues in the conserved domains of glutaminases is needed to identify their role

in enzyme catalysis. Overall, this *in silico* analysis can be considered significant for the genetic engineering of glutaminases in light of their application in food and pharmaceutical industries as well as cancer therapy.

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Conflict of Interest: None declared.

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