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Functional co-evolutionary study of glucosamine-6-phosphate synthase in mycoses causing fungi

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Abstract:
Invasive fungal opportunistic infections or mycoses have been on the rise with increase in the number of immuno-compromised patients accounting for associated high morbidity and mortality rates. The antifungal drugs are not completely effective due to increased resistance and varied susceptibility of fungi. Hence, the functional diversification study of novel targets has to be carried out. The enzyme glucosamine-6-phosphate synthase [EC 2.6.1.16], a novel drug target, catalyzes the rate-limiting step of the fungal cell-wall biosynthetic pathway, comprising four conserved domains, two glutaminase and sugar-isomerising (SIS) domains with active site. The amino acids within these domains tend to mutate simultaneously and exert mutual selective forces which might result in untoward fungal adaptations that are fixed through random genetic drift over time. The current study is an attempt to investigate such 'non-independent' coevolving residues which play critical functional and structural role in the protein. Residues with Shannon entropy <=1 (calculated by the Protein Variability Server) were considered and subsequently, positional correlations were estimated by InterMap3D 1.3 server. It was observed that majority of coevolving pairs of first SIS domain involved interactions with hydrophobic leucine and found to be spatially coupled in 3-dimensional structure of the enzyme. The coevolving groups of *Aspergillus niger* and *Rhizopus oryzae* species might play a role in drug resistance. Such coevolutionary analysis is important for understanding the receptor-ligand interactions and effective drug designing.

Keywords: Glucosamine-6-phosphate synthase, mycoses, Shannon entropy, coevolution, entropy dependency

Background:
Mycoses or invasive fungal infections such as Candidiasis, Aspergillosis, Histoplasmosis, Coccidioidomycosis, Blastomycosis, Penicilliosis, Cryptococcosis and Zygomycosis have been on the rise since the past two decades. They are caused by fungi belonging to either of the phyla, Ascomycetes, Basidiomycetes and Zygomycetes. The fungi afflict patients mostly suffering from immunocompromised diseases **[1]** and the existing drugs have limited antifungal spectrum due to increased resistance and varied susceptibility of fungi. Hence, it is imperative to search for novel antifungal drug targets against which an effective lead compound can be generated. The enzyme glucosamine-6-phosphate (G-6-P) synthase [**EC 2.6.1.16**] acts as a potential antifungal drug target which catalyzes the rate-limiting step of the fungal cell-wall biosynthetic pathway **[2]**. It consists of four conserved domains, two glutaminase (GATase) and sugar-isomerising (SIS) domains with active sites. These domains are highly conserved and functional across the mycoses-causing fungi as detected from their multiple sequence alignments (MSA) and variability analyses within the consensus sequences **[3]**. However, there are probabilities that within these domains, the amino acids might get mutated simultaneously. Identification of such 'non-independent evolution' of residues can highlight the interactions and subsequent functions among them and this motivated us to carry out co-evolutionary study of these domains at the molecular level.

Methodology:
20 amino acid sequences of G-6-P synthase for mycoses-causing fungi (**Table 1, see Supplementary material**) as listed in the World Health Organization were retrieved and compiled from

Broad Institute of MIT and Harvard (http://www.broadinstitute.org/), DOE Joint Genome Institute (http://genome.jgi-psf.org/programs/fungi/index.jsf) and NCBI fungal databases (http://www.ncbi.nlm.nih.gov/projects/genome/guide/fungi/). These sequences were manually separated according to the protein families predicted by PFAM and the variability of the individual domains was estimated by the Shannon entropy (H) analyses **[4]** using the Protein Variability Server. Subsequently, intra-domain co-evolutionary parameters were averaged out throughout the MSA by InterMap3D 1.3 server **[5]**. The intersection between predictions of 3 methods *viz.* MI/Entropy **[6]**, entropy dependency (RCW-MI) **[7]** and dependency **[8]** were selected to obtain top 10 coevolving pairs. Only those coevolving groups with one or both the residues having H<=1 were taken into consideration.

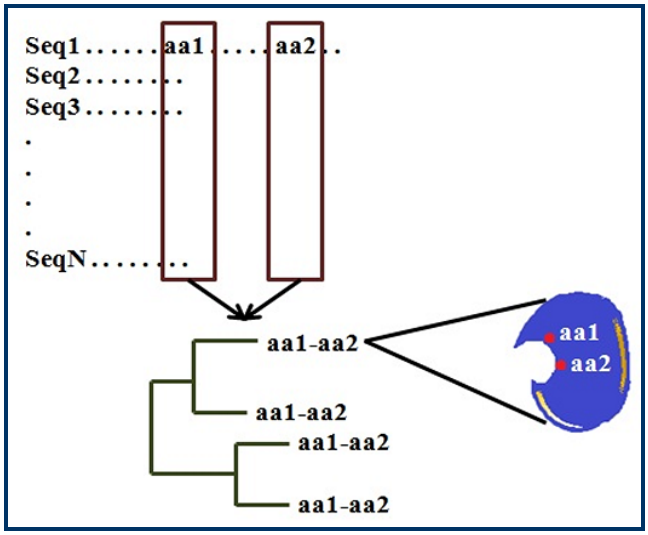

Figure 1: Representation of intra-domain co-evolutionary pair of amino acid (aa) 1 and 2 which corresponds to spatially coupled sites at active site of the enzyme.

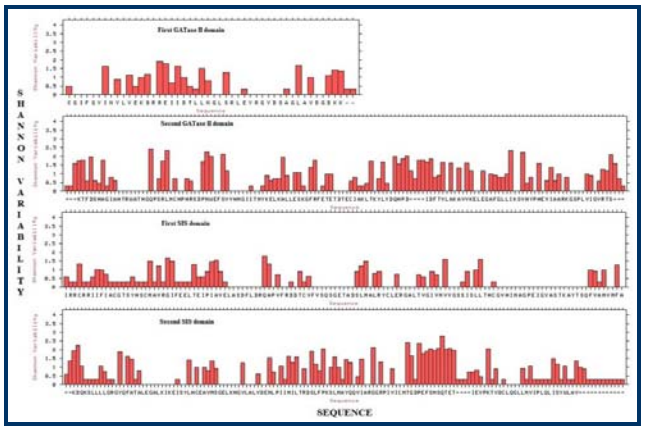

Figure 2: Shannon entropy analyses of all domains of G-6-P synthase of mycoses-causing fungi. X-axis represent the sequence of domains, Y-axis represent their Shannon variability.

Discussion:

GATase II and SIS domains are the conserved protein families of G-6-P synthase obtained from PFAM across mycoses-causing fungi. These domains specify the active sites of the enzyme which are flexible for mutations. Single mutation can be lethal, but if amino acids co-evolve simultaneously, the deleterious effect of mutation can be neutrally fixed through random genetic drift over a period of time which ideally has no effect upon the fitness of the organism. The co-evolutionary pairs exert mutual evolutionary pressures on each other leading to adaptations such as antifungal drug resistance or varied susceptibility of fungi. Hence, investigation of coevolving pairs becomes critical for better antifungal drug designing. **Figure 1** represents co-evolutionary pair within a domain where 2 amino acids have been mutated; concurrently during evolution they tend to play a significant role in the structure and function of the enzyme. To begin with Shannon entropy (H) analyses, one of the most sensitive tool, was performed to compute variability within the domains (**Figure 2**) and residues with H<=1 have been considered because they represent highly conserved positions. Subsequently, InterMap3D 1.3 server **[5]** detected intra-domain co-evolutionary pairs (**Figure 3**) and the same pair of residues repeatedly arose from the phylogenetic tree (data not shown), implying true and strong co-evolution. The coevolving pairs having either one or both the highly conserved amino acid residues with H<=1 as shown (**Supplementary material Table 2**).

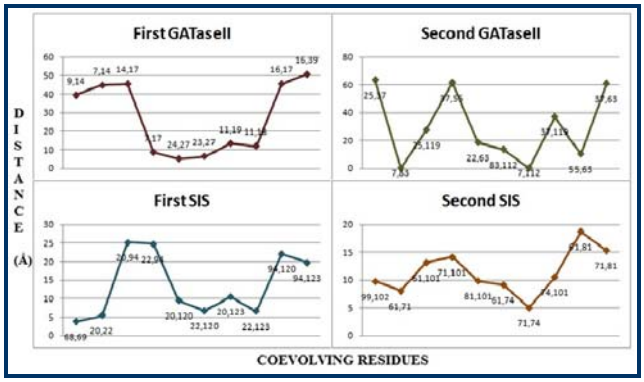

Figure 3: Coevolving pairs of all domains of G-6-P synthase of mycoses-causing fungi. X-axis represent the coevolving residues, Y-axis represent the distance (Å) between them.

In the first GATase II domain, the coevolving pair at positions 9 and 14 of *Aspergillus niger2* (*i.e.* Y-N) is not present along with the other *Aspergillus* species (Y-D). *Rhizopus oryzae1* (N-A) is separated from the rest of the zygomycetes (N-S) at positions 24 and 27. In the case of first SIS domain, 50% of the results show *A. niger2* along with the other *Aspergillus* species. It is possible that the coevolving residues of *A.niger2* and *R. oryzae1* might have an additional functional role *viz.* increased resistance against antifungal drugs. The result is in accordance with our previous study **[3]**. Moreover, most of the coevolving pairs of the first SIS domain show interactions with the hydrophobic amino acid, leucine. It might be suitable as one of the antifungal drug target sites. Coevolving pairs were obtained for the second GATase II and SIS domains as well, but none of the amino acids had H<=1 because the co-evolutionary residues were lying within slightly variable regions which are generally comprised of loop portions of an enzyme structure and known to be highly mutable. Consequently, targeting to such sites would not be a very good choice. It is also interesting to note that coevolving pairs have mostly either hydrophobic-hydrophobic

or hydrophobic-hydrophilic type of interactions, directing the nature of the antifungal drugs. Furthermore, in the 3-dimensional structure, the coevolving residues are closer to each other, *i.e.* they are spatially coupled as shown in **Figure 4**. Thus, co-evolutionary relationships might be crucial for carrying out enzymatic functions or maintaining its structure, but the reverse is not true. Hence, it is vital to consider coevolving positions for stronger receptor-ligand interactions to come up with an improved lead compound.

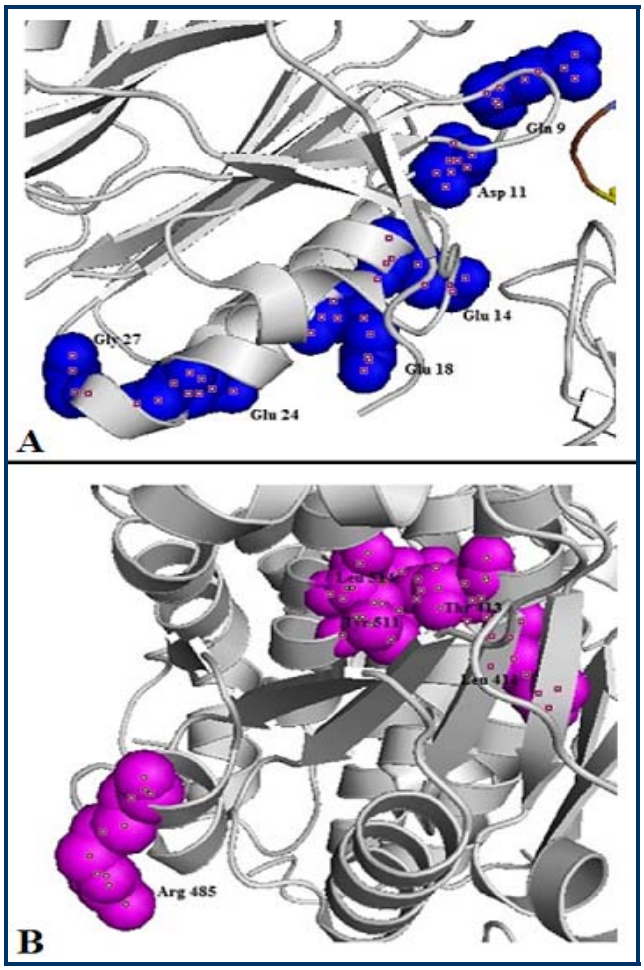

Figure 4: Spatially coupled coevolving groups identified in the domains of G-6-P synthase of mycoses-causing fungi (Pymol software used for visualization). a) Domain First GATase I (PDBID: 1XFG) with coevolving aminoacids Gln9 & Glu14, Glu24 & Gly27, and Asp11 & Glu18. b) Domain First SIS (PDBID: 2PUW) with coevolving aminoacids Leu411 & Arg485, Thr413 & Arg485, Leu411 & Tyr511, Thr413 & Tyr511, Leu411 & Leu514, Thr413 & Leu514, Arg485 & Tyr511, and Arg485 & Leu514.

Conclusion:

Co-evolutionary study of functional domains within G-6-P synthase of mycoses-causing fungi was conducted at the molecular level to better understand the factors that influence the functional and structural aspects of the enzyme, particularly the increasing fungal resistance against drugs. Admissible changes at one residue alter the selective forces on other amino acids; referred to as coevolving pairs. These coordinated neutral mutations help in the functional annotations and are spatially coupled in the 3-dimensional structure of the enzyme. The current study evaluated the coevolving pairs within the conserved domains of the enzyme subsequent to estimation of their Shannon entropies. This study implies the functional diversification of residues at active sites which might be the cause for antifungal drug resistance or other untoward fungal adaptations in the future. Such analyses could assist in better computer-aided drug designing of antifungal drugs.

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Supplementary material:

Table 1: List of mycoses-causing fungi used in the current study as listed in the World Health Organization.

Ascomycetes	Basidiomycetes	Zygomycetes
Candida albicans (713aa)	*Cryptococcus neoformans* (706aa)	*Rhizopus oryzae_1* (688aa)
Candida tropicalis (686aa)		*Rhizopus oryzae_2* (688aa)
Candida dubliniensis (711aa)		*Mucor circinelloides* (688aa)
Candida glabrata (723aa)		*Phycomyces blakesleeanus* (692aa)
Aspergillus nidulans (898aa)		
Aspergillus fumigatus (694aa)		
Aspergillus flavus (693aa)		
Aspergillus niger_1 (694aa)		
Aspergillus niger_2 (702aa)		
Aspergillus clavatus (694aa)		
Coccidioides immitis (716aa)		
Penicillium chrysogenum (694aa)		
Penicillium marneffei (694aa)		
Blastomyces dermatitidis (694aa)		

Table 2: Coevolving pairs of amino acids identified within the domains of G-6-P synthase of mycoses-causing fungi. AF- *Aspergillus flavus*, AN1- *A. niger1*, AN2- *A. niger2*, Afu- *A. fumigatus*, AC- *A. clavatus*, Anid- *A. nidulans*, PM- *Penicillium marneffei*, PC- *P. chrysogenum*, BD- *Blastomyces dermatitidis*, HC- *Histoplasma capsulatum*, CI- *Coccidioides immitis*, CA- *Candida albicans*, CD- *C. dubliniensis*, CT- *C. tropicalis*, CG- *C. glabrata*, PB- *Phycomyces blakes*, CN- *Cryptococcus neoformans*, RO1- *Rhizopus oryzae1*, RO2- *R. oryzae2*, MC- *Mucor circinelloides*.

Position	Coevolving residues	Fungi	Frequency
Coevolving residues in first GATase II domain			
9 & 14	Y-D	AN1,CI,BD,HC,PM,PC,AF,Afu,AC,Anid,PB	0.611
	F-S	RO1,RO2,MC,CA,CD	0.278
	Y-S	CG	0.056
	Y-N	AN2	0.056
24 & 27	N-S	AN1,CI,BD,HC,PM,PC,RO2,MC,AN2,AF,Afu,AC,Anid	0.722
	E-Q	CA,CD	0.111
	N-A	RO1,PB	0.111
	D-K	CG	0.056
11 & 18	V-I	AN1,CI,PM,PC,CG,CA,CD,AN2,AF,Afu,AC,Anid,PB	0.722
	C-V	RO1,RO2,MC	0.167
	I-I	BD,HC	0.111
Coevolving residues in first SIS domain			
20 & 94	M-L	AF,AN1,Afu,AC,PM,BD,Anid,HC,CI,PC	0.526
	L-R	CT,CD,CA,RO1,RO2,MC,PB,CG	0.421
	V-L	AN2	0.053
22 & 94	V-L	AF,AN1,Afu,AC,PM,BD,Anid,HC,CI,PC,AN2	0.579
	T-R	CT,CD,CA,RO1,RO2,MC,PB,CG	0.421
20 & 120	M-F	AF,AN1,Afu,AC,PM,BD,Anid,HC,CI,PC	0.526
	L-Y	CT,CD,CA,RO1,RO2,MC,PB,CG	0.421
	V-F	AN2	0.053
22 & 120	V-F	AF,AN1,Afu,AC,PM,BD,Anid,HC,CI,PC,AN2	0.579
	T-Y	CT,CD,CA,RO1,RO2,MC,PB,CG	0.421
20 & 123	M-M	AF,AN1,Afu,AC,PM,BD,Anid,HC,CI,PC	0.526
	L-L	CT,CD,CA,RO1,RO2,MC,PB,CG	0.421
	V-M	AN2	0.053
22 & 123	V-M	AF,AN1,Afu,AC,PM,BD,Anid,HC,CI,PC,AN2	0.579
	T-L	CT,CD,CA,RO1,RO2,MC,PB,CG	0.421
94 & 120	L-F	AF,AN1,Afu,AC,PM,BD,Anid,HC,CI,PC,AN2	0.579
	R-Y	CT,CD,CA,RO1,RO2,MC,PB,CG	0.421
94 & 123	L-M	AF,AN1,Afu,AC,PM,BD,Anid,HC,CI,PC,AN2	0.579
	R-L	CT,CD,CA,RO1,RO2,MC,PB,CG	0.421