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Inference of *Aspergillus fumigatus* Pathways by Computational Genome Analysis: Tricarboxylic Acid Cycle (TCA) and Glyoxylate Shunt

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Abstract *Aspergillus fumigatus* is one of the most common fungi in the human environment, both in-doors and out-doors. It is the main causative agent of invasive aspergillosis, a life-threatening mycosis among immunocompromised patients. The genome has been sequenced by an international consortium, including the Wellcome Trust Sanger Institute (U.K.) and The Institute for Genomic Research (TIGR, U.S.A.), and a ten times whole genome shotgun sequence assembly has been made publicly available. In this study, we identified tricarboxylic acid (TCA) cycle enzymes of *A. fumigatus* by comparative analysis with four other fungal species. The open reading frames showed high amino acid sequence similarity with the other fungal citric acid enzymes and well-conserved functional domains. All genes present in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, and *Neurospora crassa* were also found in *A. fumigatus*. In addition, we identified four *A. fumigatus* genes coding for enzymes in the glyoxylate shunt, which may be required for fungal virulence. The architecture of multi-gene encoded enzymes, such as isocitrate dehydrogenase, 2-ketoglutarate, succinyl-CoA synthetase, and succinate dehydrogenase was well conserved in *A. fumigatus*. Furthermore, our results show that genes of *A. fumigatus* can be detected reliably using GlimmerM.

Key words: *Aspergillus fumigatus*, comparative analysis, TCA cycle, gene prediction

In the past 20 years, the ascomycetous fungus *Aspergillus fumigatus* has gone from being a saprophytic fungus of minor interest to becoming one of the most important fungal pathogens of humans. Its natural habitat is the soil, in which the fungus grows on organic debris. *Aspergillus fumigatus* reproduces by producing large numbers of airborne conidiospores [23]. These spores are small enough to reach the lung alveoli. Conidia inhaled by susceptible patients can lead to life-threatening invasive pulmonary aspergillosis [15]. The main reason for the rise in systemic infections lies in the steadily growing number of immunocompromised individuals, the main risk group for such infections. Although *A. fumigatus* only makes up a small proportion of all aerial spores, around 0.3% in the air of one particular hospital, it causes roughly 90% of invasive aspergillosis cases [3]. This suggests that *A. fumigatus* possesses certain factors that allow it to become an opportunistic human pathogen in immunocompromised patients. In recent years, great progress has been made in understanding the molecular genetics of *A. fumigatus* [3]. The genome of *A. fumigatus* is currently being sequenced by an international consortium. Based on preliminary sequencing data, the genome of *A. fumigatus* is estimated to be 30 Mb (http://www.sanger.ac.uk) [9].

In this study, we have concentrated on well-known metabolic pathways such as the tricarboxylic acid (TCA) cycle and the glyoxylate shunt. We have attempted a reconstruction of this pathway in *A. fumigatus* by carrying out a comparative genomic analysis using four other fungal genomes.

MATERIALS AND METHODS

Sequence Databases

TCA cycle enzymes from the fungal species *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Neurospora crassa* were retrieved from several databases (geneDB, http://www.genedb.org; CandidaDB, http://genolist.pasteur.fr/CandidaDB; NeurosporaDB, http://www-genome.

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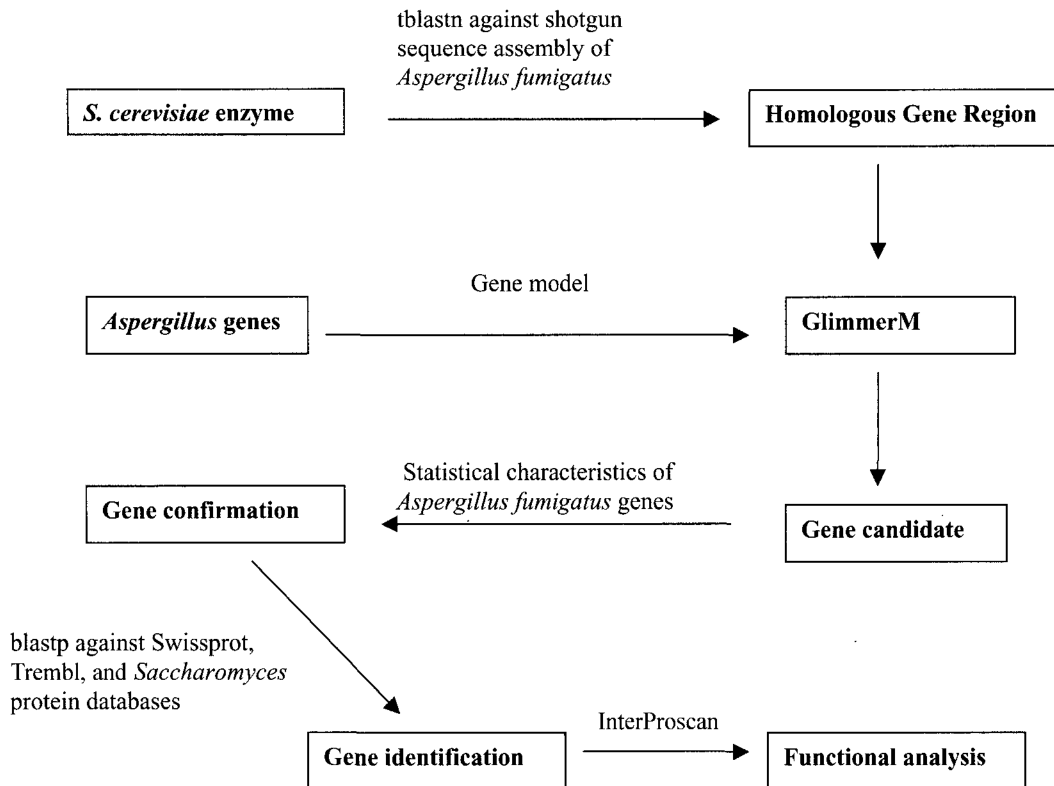

Fig. 1. An overview of the process for identifying TCA cycle and glyoxylate shunt genes in the *Aspergillus fumigatus* genome.

wi.mit.edu/annotation/fungi/neurospora; SPTR, http://srs.ebi.ac.uk).

The ten-fold whole genome shotgun sequence assembly of *A. fumigatus* was obtained from the TIGR website at http://www.tigr.org. The TIGR BLAST search engine runs the WU-BLAST 2.0 program (http://blast.wustl.edu). For similarity searches, the tblastn program was used and the E-value cutoff used to assign homologues was 1-e6.

Gene Prediction by GlimmerM

The known TCA enzymes of the four selected fungi were used as queries for searching against the *A. fumigatus* genome. The regions of the *A. fumigatus* genome possessing high similarity with the TCA enzymes were used as the input for GlimmerM (http://www.tigr.org) [27]. The basis of GlimmerM is a dynamic programming algorithm that considers all combinations of possible exons for inclusion in a gene model and chooses the best of these combinations. GlimmerM has been trained by the Eukaryotic Annotation team at TIGR for use with the *Aspergilli* using a training set of 210 *Aspergillus* genomic sequences validated by mRNA or validated by protein matches. This set contained 190 complete genes and 76 partial genes (only 13 were from *A. fumigatus*), consisting of 39 intronless genes, 722 exons and 532 introns, 558 acceptor sites and 555 donor sites. The genes predicted by GlimmerM were edited manually if necessary, according to the statistical data of *A. fumigatus* genes produced by Anderson *et al.* [1].

Validation of Predicted Genes (Fig. 1)

For validation of the predicted TCA genes of *A. fumigatus*, a bi-directional best-hit analysis was performed, using the polypeptide sequences of the predicted *A. fumigatus* ORFs as queries for blastp searches of the protein databases at the Swiss Institute of Bioinformatics (http://www.ch.embnet.org; http://SwissProt/TrEMBL/TrEMBL_NEW) and of the *Saccharomyces* protein database at SGD (http://genome-www.stanford.edu/Saccharomyces). A functional analysis of the predicted genes was conducted using the polypeptide sequences of the predicted *A. fumigatus* ORFs as query sequences for InterProScan (http://www.ebi.ac.uk/interpro/scan.html).

RESULTS

TCA Cycle Enzymes in Four Fungal Species

In order to identify TCA cycle genes in *A. fumigatus*, we surveyed various databases for the enzyme or gene from four fungal species (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans* and *Neurospora crassa*). Huynen *et al.* [14] graphically

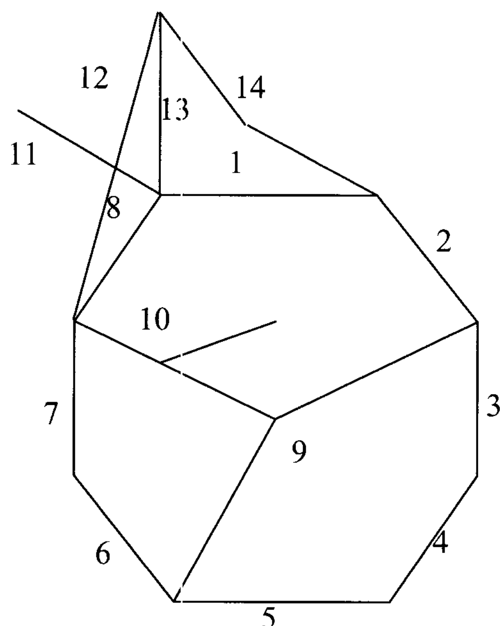

Fig. 2. A graphical representation of the reactions of the TCA cycle, including the connections with pyruvate and phosphoenolpyruvate, and of the glyoxylate shunt (adapted from Huynen *et al.* [14]).

The enzymes with their EC numbers are as follows: 1, citrate synthase (4.1.3.7); 2, aconitase (4.2.1.3); 3, isocitrate dehydrogenase (1.1.1.41, 1.1.1.42); 4, 2-ketoglutarate dehydrogenase (1.2.4.2, 2.3.1.61); 5, succinyl-CoA synthase (6.2.1.4); 6, succinate dehydrogenase (1.3.5.1); 7, fumarase (4.2.1.2); 8, malate dehydrogenase (1.1.1.37); 9, isocitrate lyase (4.1.3.1); 10, malate synthase (4.1.3.2); 11, PEP carboxykinase (4.1.1.49); 12, malic enzyme (1.1.1.38); 13, pyruvate carboxylase (6.4.1.1); 14, pyruvate dehydrogenase (1.2.4.1, 2.3.1.12, 1.8.1.4).

represented the reactions of the TCA cycle, including the connections with pyruvate and phosphoenolpyruvate, and the glyoxylate shunt, which results in a total of 14 reactions (Fig. 2). They reported that most organisms had an incomplete TCA cycle when they examined the genomes of unicellular organisms including those of four Archaea, 14 Bacteria, and one Eukaryote. In incomplete cycles, the last part of the oxidative cycle (steps 6–8 in Fig. 2), leading from succinate to oxaloacetate, is the most highly conserved, whereas the initial steps (steps 1–3), from acetyl CoA to 2-ketoglutarate, show the least conservation. The eight enzymes of the TCA cycle are encoded by at least 15 different nuclear genes in *S. cerevisiae* [21]. Four enzymes, citrate synthase, aconitase, fumarase, and malate dehydrogenase are coded by the single genes, *CIT1*, *ACO1*, *FUM1*, *MDH1*, respectively [28, 11, 29, 18]. The other four enzymes are composed of subunits encoded by more than one gene. The NAD^+-dependent isocitrate dehydrogenase (NAD-IDH) is an octamer composed of four *IDH1* and four *IDH2* subunits encoded by the *IDH1* and *IDH2* genes [6, 7]. The 2-ketoglutarate (2-oxoglutarate) dehydrogenase complex (KGDC) contains three different subunits encoded by the *KGD1*, *KGD2*, and *LPD1* genes [24–26]. Interestingly, the lipoamide dehydrogenase protein that is encoded by the *LPD1* gene is also a component of the pyruvate dehydrogenase and branched chain amino acid dehydrogenase complexes. Succinyl-CoA ligase is a heterodimer composed of an α-subunit encoded by the *LSC1* gene and a β-subunit encoded by the *LSC2* gene [20]. Ubiquinone:succinate dehydrogenase is an integral membrane protein composed of four subunits encoded by the *SDH1* through *SHD4* genes [4, 5, 8, 16].

The architecture of the TCA cycle enzymes of *S. cerevisiae* is well conserved in other fungi, such as *S. pombe*, *C. albicans*, and *N. crassa* (Table 1). In the case of *S. pombe*, malate synthase and phosphoenolpyruvate carboxykinase (PEPCK) are absent. PEPCK converts oxaloacetate to phosphoenolpyruvate (PEP), which is then converted to hexose phosphates, and is necessary for growth on compounds that are metabolized via TCA cycle intermediates. Therefore, *S. pombe* cannot utilize efficiently nonfermentable carbon sources such as acetate and ethanol. However, *S. pombe* might bypass PEPCK by the joint action of malic enzyme and PEP kinase that converts pyruvate to PEP, as has been described in *E. coli* mutants deficient in PEPCK [12].

In *C. albicans*, the subunit *SDH3* of succinate dehydrogenase is missing in the *Candida* proteome database. Subunits *SDH3* and *SDH4* are integral membrane proteins, which have low percentage identities amongst themselves, while subunits *SDH1* and *SDH2* are hydrophilic proteins with high percentage identities between species. A tblastn search with *S. cerevisiae SDH3* against the *C. albicans* genome (assembly 6, http://www-sequence.stanford.edu/group/candida) resulted in a hit with a high level of similarity (46% identity in a 154-amino-acid overlap on contig_6-2051). Therefore, it looks like this enzyme is present in the genome and that it was overlooked when the *Candida* proteome database was generated.

Neurospora crassa TCA cycle genes identified by their best bi-directional hit to the yeast gene set are represented by their gene locus number (NeurosporaDB) in those cases where the individual TCA cycle enzyme is not available in the SPTR database (Table 1).

Identification of TCA Cycle and Glyoxylate Genes in the *A. fumigatus* Genome

The *S. cerevisiae* enzymes of the TCA cycle and glyoxylate shunt pathways were used as query sequences in a blast analysis to identify the homologous genomic regions in *A. fumigatus*. The next step was to identify precisely the protein-coding regions within these homologous regions (Fig. 1). The prediction of protein-coding genes within DNA sequences in eukaryotes is not an easy task. This could be due to the fact that the coding density is low (probably as low as 2% in human, though fungal genomes

Table 1. The TCA cycle and glyoxylate shunt enzymes in four fungal species (in *N. crassa* the gene identified by a best bi-directional blast hit to the yeast protein is represented by a gene locus number with the prefix of NCU).

Enzymes (no. in brackets refers to Fig. 2)	Genes	Species			
		S. cerevisiae	*S. pombe*	*C. albicans*	*N. crassa*
Citrate synthase (1)					
EC 4.1.3.7	**CIT1**	YNR001C	SPAC6C3.04	CIT1	CIT1
Aconitase (2)					
EC 4.2.1.3	**ACO1**	YLR304C	SPAC24C9.06c	ACO1	B11A5.190
Isocitrate dehydrogenase (3)					
EC 1.1.1.41	**IDH1**	YNL037C	SPAC11G7.03	IDH1	NCU00775.1
	IDH2	YOR136W	SPBC902.05c	IDH2	NCU07697.1
EC 1.1.1.42					
	IDP1	YDL066W	SPAC6G10.08	IDP1	NCU03857.1
2-Ketoglutarate dehydrogenase (4)					
EC 1.2.4.2	**KGD1**	YIL125W	SPBC3H7.03c	KGD1	B8B20.370
EC 2.3.1.61	**KGD2**	YDR148C	SPBC776.15c	KGD2	NCU02438.1
Succinyl-CoA synthetase (5)					
6.2.1.4	**LSC1**	YOR142W	SPAC16E8.17c	LSC1	8D4.130
	LSC2	YGR244C	SPCC1620.08	LSC2	B9J10.140
Succinate dehydrogenase (6)					
EC 1.3.5.1	**SDH1**	YKL148C	SPAC1556.02c	SDH1	NCU04768.1
	SDH2	YLL041C	SPAC140.01	SDH2	NCU00959.1
	SDH3	YKL141W	SPCC330.12c	NP	NCU07756.1
	SDH4	YDR178W	SPBP23A10.16	SDH4	NCU03031.1
Fumarase (7)					
EC 4.2.1.2	**FUM1**	YPL262W	SPCC18.18c	FUM1	NCU10008.1
Malate dehydrogenase (8)					
EC 1.1.1.37	**MDH1**	YKL085W	SPCC306.08c	MDH1	B2F7.120
Isocitrate lyase (9)					
EC 4.1.3.1	**ICL1**	YER065C	SPBC1683.11c	ICL1	ACU3
Malate synthase (10)					
EC 4.1.3.2	**MLS1/**	YNL117W	NP	MLS1	ACU9
	DAL7	YIR031C			
PEP carboxykinase (11)					
EC 4.1.1.49	**PCK1**	YKR097W	NP	PCK1	NCU09873.1
Malic enzyme (12)					
EC 1.1.1.38	**MAE1**	YKL029C	SPCC794.12c	MAE1	NCU02906.1
Pyruvate carboxylase (13)					
EC 6.4.1.1	**PYC1/**	YGL062W	SPBC17G9.11c		
	PYC2	YBR218C		PYC2	NCU02505.1
Pyruvate dehydrogenase (14)					
EC 1.2.4.1	**PDA1**	YER178W	SPAC26F1.03	PDA1	NCU06482.1
	PDB1	YBR221C	SPBC30D10.13c	PDB1	B24P7.220
EC 2.3.1.12	**LAT1**	YNL071W	SPCC794.07	LAT1	MRP3
EC 1.8.1.4	**LPD1**	YFL018C	SPAC1002.09c	LPD1	NCU02407.1

NP: gene not present.

have densities of at least 40%) and the presence of introns within relatively short coding regions. Various different, but weak signals have to be combined such as GC bias, splice sites, and translational start and stop sites. Different knowledge-based methods complemented by similarity searches are applied to utilize them [13]. The commonly used methods for eukaryotic gene prediction depend on training a computer program to recognize sequences that are characteristic of known exons in genomic DNA sequences. The GlimmerM program [27] was trained with the *Aspergillus* training set for Glimmer provided by TIGR in order to enable the prediction of the positions of exons in genomic sequences and their arrangement into a predicted gene structure. The trained GlimmerM program was used to predict the positions of genes within regions of the *A. fumigatus* genome that were identified by blast

Table 2. The predicted TCA cycle and glyoxylate shunt genes in the *A. fumigatus* genome and the results of their best hit using blastp against the *Saccharomyces* proteome and of InterProScan.

Genes	Contig number/size of predicted protein	Blast Results to yeast proteome: E-value/ percent identity; percent similarity in amino acid overlap	InterPro Accession Number	Function
CIT1	4892(75,701-76,881)/344 aa	1.6e-124/ 50%; 66%; in 340 aa	IPR002020	Citrate synthase
CIT2	4899(510,445-502,823)/461aa	7.8e-94/ 48%; 64%; in 375 aa	IPR002020	Citrate synthase
ACO1	4941(188,748-191,428)/797 aa	6.1e-306/ 74%; 85%; in 726 aa	IPR006248	Aconitase
IDH1	4821(47,436-48,831)/293 aa	4.0e-62/ 58%; 74%; in 187 aa	IPR001804	Oxireductase (*isodh*)
IDH2	4836(466,111-466,779)/164 aa	5.0e-50/ 61%; 76%; in 161 aa	IPR001804	Oxireductase (*isodh*)
IDP1	4085(94,166-95915)/458 aa	9.0e-138/ 66%; 78%; in 379 aa	IPR001804	Oxidoreductase (*isodh*)
KGD1	4839(62,563-65586)/943 aa	0.0/ 67%; 80%; in 818 aa	IPR001017	Oxidoreductase (*2oxo_dh_E1*)
KGD2	4898(409,059-410,159)/212 aa	2.8e-59/ 72%; 81%; in 126 aa	IPR001078	Acyltransferase
LSC1	4944(69,823-70,677)/217 aa	3.7e-38/ 42%; 54%; in 186 aa	IPR003781	Succinyl-CoA ligase, alpha unit
LSC2	4882(550,139-550,827)/185 aa	2.7e-42/ 50%; 70%; in 163aa	IPR003135	Succinyl-CoA ligase, beta unit
SDH1	4871(155,655-157,673)/549 aa	1.7e-205/ 76%; 86%; in 484 aa	IPR003953	Electron transfer flavoprotein
SDH2	4938(931,926-932,587)/199 aa	9.3e-49/ 65%; 82%; in 132 aa	IPR006058	Iron-sulfur electron transfer carrier
SDH3	4938(778,700-779,503)/205 aa	9.3e-17/ 32%; 52%; in 126 aa	IPR000701	Succinate dehydrogenase
SDH4	4942(154,408-155,556)/145 aa	6.3e-11/ 35%; 56%; in 98 aa	-	-
FUM1	4899(821,079-821,530)/132 aa	3.8e-43/ 67%; 80%; in 128 aa	IPR000362	Fumarate lyase
MDH1	4901(83,598-84,888)/317 aa	6.3e-101/ 62%; 84%; in 194 aa	IPR001252	Malate dehydrogenase
MDH2	4899(34,119-34,866)/231 aa	2.3e-35/ 47%; 62%; in 137 aa	IPR001252	Malate dehydrogenase
ICL1	4865(388,928-389,710)/260 aa	2.1e-81/ 59%; 75%; in 257 aa	IPR000918	Isocitrate lyase
MLS1	4899(515,571-517,328)/535 aa	6.0e-157/ 67%; 79%; in 429 aa	IPR001465	Malate synthase
PCK1	4963(338,231-339,721)/476 aa	3.0e-178/ 69%; 81%; in 449 aa	IPR001272	PEP carboxykinase
MAE1	4937(62,499-63,875)/374 aa	1.6e-69/ 44%; 60%; in 360 aa	IPR001891	Malic enzyme
PYC2	4826(501,032-504,639)/1172 aa	0.0/ 66%; 78%; in 1130 aa	IPR005930	Pyruvate carboxylase
PDA1	4933(440,482-441,723)/370 aa	6.8e-133/ 65%; 81%; in 355 aa	IPR001017	Oxidoreductase (*E1_dehydrog*)
PDB1	4889(40,888-41,723)/238 aa	2.6e-60/ 60%; 72%; in 186 aa	IPR005475	Transketolase
LAT1	4901(89,108-90,329)/366 aa	4.1e-79/ 53%; 68%; in 248 aa	IPR001078	Acyltransferase
LPD1	4942(37,115-38,907)/554 aa	2.3e-36/ 27%; 45%; in 459 aa	IPR001327	Disulfide oxidoreductase (*pyr_redox*)

Abbreviations: *isodh*, Isocitrate/isopropylmalate dehydrogenase; *2oxo_dh_E1*, 2-oxoglutarate dehydrogenase E1 component; *E1_dehydrog*, dehydrogenase E1 component; *pyr_redox*, pyridine nucleotide-disulfide oxidoreductase.

searching with other fungal TCA cycle enzymes. Figure 3 shows the exon size distribution for these predicted genes. The exon size ranges from 8 to 3,473 bp with an average of 369 bp over 97 exons. These data were similar to those from Anderson *et al.* [1]. The average size of the introns is 85 bp, which is smaller than that reported for *N. crassa* (103 bp) [22]. The average number of introns per gene is 2.5, whereas Deutsch and Long reported that *Aspergillus* species have a mean of 3.1 introns per gene [10]. Most of the introns in this dataset had canonical splice site sequences. Ninety-four percent of the introns have a recognizable consensus lariat sequence (CTRAY where R is purine and Y a pyrimidine) and this sequence is similar to that found in *N. crassa* [22]. Although only a small number of genes were analyzed, it can be concluded that the genes in *A. fumigatus* have a similar structure to those in other fungi.

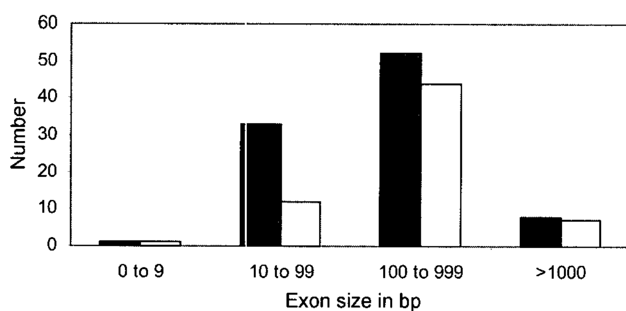

Fig. 3. The distribution of exon sizes from the predicted TCA cycle genes (black bar) and from experimentally determined non-TCA genes of *A. fumigatus* (white bar, taken from Anderson *et al.* [1]).

The identification of the predicted TCA cycle genes of *A. fumigatus* was validated by performing a reciprocal blastp search against the *Saccharomyces* proteome database (Fig. 1). Using the predicted polypeptide sequence encoded by the *A. fumigatus* genes as query sequence, all of the known TCA cycle proteins of *S. cerevisiae* were identified

(Table 2). The predicted TCA cycle proteins of *A. fumigatus* also showed high similarity to the amino acid sequences encoded by *A. nidulans* TCA cycle genes, such as isocitrate dehydrogenase (*IdpA*), malate synthase (*Acu*), and PEP carboxykinase (*AcuF*) (data not shown).

Each predicted TCA cycle protein was further characterized by a computational functional analysis using InterProScan. All of the predicted proteins, except one, had a significant hit to a protein family or domain and so could be classified by this function or functional domain (see column 6 of Table 2). The exception was contig 4,942 (154, 408- 155, 556), which contains the gene for the membrane anchor protein of succinate dehydrogenase. There is also no match for the other fungal *SDH4* proteins using InterProScan [Pfam is included in InterPro]. It must be presumed that the low level of sequence identity between *SDH4* proteins has hindered the creation of a protein family domain.

Aspergillus fumigatus has a complete glyoxylate shunt pathway, in addition to the TCA cycle (Table 2). Lorenz and Fink reported that glyoxylate-specific enzymes, such as *ICL1* and *MLS1* are required for fungal virulence [17]. The *A. fumigatus ICL1* homologue has an identity of 59% to the *S. cerevisiae* protein, whereas the *MLS1* homologue has an identity of 65%.

DISCUSSION

The application of the basic paradigm of comparative genomics (proteins with similar sequence are likely to have similar function) showed that there was conservation in parts of the central metabolism (the TCA cycle and glyoxylate shunt pathways) in five fungal species including *A. fumigatus*. The computationally predicted genes in this study should be confirmed by direct experimentation. The approach described here could be applied to other pathways, such as glycolysis and lipid metabolism, because it is unlikely that isolated proteins evolve, for example, through the duplication of their genes without their contextually-linked proteins duplicating and evolving as well. Gene duplication can have an impact on the co-regulation of genes, gene order, flux of metabolites, and the other parameters. It has been reported that *A. fumigatus,* like *S. cerevisiae*, has duplicated genes, such as the *RHO* and chitinase genes [2, 19]. GlimmerM is a gene finder developed specifically for small eukaryotes with a gene density of around 20%. The gene density of *A. fumigatus* is much lower than that of bacteria, but is much higher than that of humans. Our results show that genes in *A. fumigatus* can be detected reliably using GlimmerM. However, the performance of GlimmerM as a gene finder for the *A. fumigatus* genome would undoubtedly be improved if an *A. fumigatus* specific training set was to be used.

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