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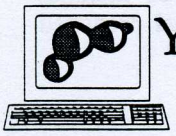

Yeast Functional Analysis Reports

Phylogenetic Classification of the Mitochondrial Carrier Family of *Saccharomyces cerevisiae*

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The screening of the open reading frames identified in the whole yeast genome has allowed us to discover 34 proteins belonging to the mitochondrial carrier family. By phylogenetic study, they can be divided into 27 subfamilies including ADP/ATP, phosphate and citrate carriers, putative oxoglutarate and GDC carriers and 22 new subfamilies. Topology predictions using the 'positive inside rule' approach have shown that the yeast carriers are similarly oriented with both extremities exposed to the cytosol. In each subfamily, a strict conservation of the charged residues in the six transmembrane α-helices is observed, suggesting a functional role for these residues and the existence of 27 functionally distinct carriers.

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KEY WORDS — yeast genome; mitochondrial carrier family; transport protein

INTRODUCTION

The mitochondrial inner membrane contains a set of intrinsic membrane carriers responsible for shuttling metabolites between cytosol and matrix. Up to now, 13 carriers have been well characterized in various organisms (Krämer and Palmieri, 1992; Palmieri, 1994). Most of these carriers transport anions (ADP/ATP, inorganic phosphate, pyruvate, glutamate, aspartate, oxoglutarate, dicarboxylate, citrate, ATP.Mg/Pi and fatty acid anions), but some transport cations or zwitterions such as ornithine, carnitine and glutamine. In addition to these carriers, the mitochondrial inner membrane may contain additional carriers required for the import of various compounds which are needed but not synthesized in the mitochondrial matrix, such as arginine, proline, thiamine-PP, glutathion, choline, various nucleotides, biotin, CoA, etc.

The amino acid sequences of five functionally characterized carriers have been determined by amino acid analysis or DNA sequencing, namely the ADP/ATP, phosphate, oxoglutarate and citrate carriers and the uncoupling protein (for reviews, see Kuan and Saier, 1993b; Walker and Runswick, 1993). All these sequences, including various isoforms, have similar structural features: a significant primary structure isology, a tripartite structure (three repeats of about 100 amino acids) and the presence of two hydrophobic segments in each repeat. For these proteins belonging to the same family, a common topological model has

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been proposed (Walker and Runswick, 1993; Walker, 1992). Each repetitive domain consists of two transmembrane α-helices connected by an extensive hydrophilic segment (A, B, C), whereas the three repeats are linked by shorter hydrophilic loops (a, b); in this model, the N- and C-terminal ends are located on the cytosolic side of the membrane opposite that of the three long hydrophilic loops. Moreover, in these proteins, a conserved sequence is often present at the C-terminal end of the first helix of the repeats: the mitochondrial energy transfer protein signature (P-X-(D/E)-X-(LIVAT)-(RK)-X-(LRH)-(LIVMFY) (Bairoch, 1993).

The features of the mitochondrial carrier family (MCF) sequences described above have allowed us to identify new members of this family. The phylogenetic tree based on the resulting sequence alignment revealed that the MCF members fall on 17 different branches, representing 17 subfamilies or clusters (Kuan and Saier, 1993a, b; Nelson *et al.*, 1993). Five clusters contain carriers of known functions (ADP/ATP, phosphate, oxoglutarate and citrate carriers, and uncoupling protein) and 12 clusters are putative carriers from various sources.

In the yeast *Saccharomyces cerevisiae*, an important model organism for the study of basic functions of the eukaryotic world, three mitochondrial carriers of known function have been sequenced so far: ADP/ATP (three isoforms), phosphate and citrate carriers (Adrian *et al.*, 1986; Lawson and Douglas, 1988; Kolarov *et al.*, 1990; Phelps *et al.*, 1991; Kaplan *et al.*, 1995). Moreover, some genes coding for yeast proteins of unknown function have been identified as members of the MCF, such as the *MRS3* and *MRS4* genes encoding suppressors of mitochondrial splicing defect (Wiesenberger *et al.*, 1991), the *ACR1* gene encoding a protein which is essential for acetyl CoA synthetase (Fernández *et al.*, 1994), the *RIM2* gene encoding a protein that is essential for mitochondrial DNA metabolism and rescues the respiratory defect of pif1 and mrs2 null mutants (Demolis *et al.*, 1993; Van Dyck *et al.*, 1995) and the *ARG11* gene encoding a putative carrier required for arginine biosynthesis (Crabeel *et al.*, 1996).

As the complete yeast genome sequence is now available (24 April 1996), it was screened to detect all genes which exhibit isology with the mitochondrial carrier family. This search revealed a total of 34 putative mitochondrial carriers. This paper reports the phylogenesis of these putative carrier sequences and shows that the yeast MCF includes 27 functional subfamilies with identical predicted transmembrane topology.

MATERIALS AND METHODS

Screening of the database for MCF members

The open reading frames (ORFs) discovered during the systematic sequencing of the whole genome of *S. cerevisiae* have been analysed in order to identify new members of the MCF using the BLAST and FASTA programs (Altschul *et al.*, 1990; Pearson and Lipman, 1988) against the SGD, EMBL, MIPS and GenBank databases. The mitochondrial energy transfer protein signature of the MCF (PS00215: mitoch-carrier) was detected using the PROSITE program based on the pp-search software and derived from the MacPattern program developed by R. Fuchs (Fuchs, 1991, 1994). The tripartite structure of the sequences was verified by the REPRO algorithm that delineates protein sequence fragments displaying similarity (Heringa, 1994).

Other data about the new ORFs of MCF have been found in YPD, SwissProt and PIR databases.

Binary comparison and alignment of amino acid sequences

All programs used were part of the GCG package, version 8.0 (Devereux *et al.*, 1984) running at the Belgian EMBnet Node facility. The binary comparison scores and alignment of the sequences have been produced by the Pileup program, using gap creation and gap extension penalties of 3 and 0·1 respectively.

The hydropathy profiles have been computed according to Kyte and Doolittle (1982) using the PeptideStructure program (Wolf *et al.*, 1987) with a window of seven amino acid residues.

Phylogenetic tree construction

A measure of distances between aligned proteins was computed with the Prodist program using maximum likelihood estimates on the Dayhoff PAM matrix (Dayhoff, 1979). The Fitch program estimates phylogenies from distance matrix data using the Fitch-Margoliach method with the default options (Fitch and Margoliash, 1967). The phylogenetic trees were constructed with the Drawtree program. All these programs are part of the PHYLIP package version 3.5c developed by

Felsenstein (1989) and available by 'anonymous ftp' from evolution.genetics.washington.edu in directory pub/phylip.

RESULTS

Screening and amino acid sequence analysis

Screening the ORFs of all the *S. cerevisiae* chromosomes with the BLAST and FASTA programs revealed some putative MCF members. These putative carriers were analysed with the PROSITE, REPRO and PeptideStructure programs to detect the main features of the mitochondrial carriers: a tripartite structure, the presence of two hydrophobic domains in each repeat supposed to be folded as transmembrane helices, and the presence of a conserved motif after the first helix of the repeats.

Table 1 lists the putative yeast mitochondrial carriers with their name in YPD and MIPS, as well as their accession numbers in PIR and GenBank. All the yeast chromosomes contain members of the MCF except chromosomes I and III. All except three of these gene products are around 300 residues long: the *YP1* and *YNG2* genes located on chromosomes XVI and XIV encode proteins with 902 and 494 residues respectively including a long N-terminal extension (533 and 230 residues respectively), while the *YFL5* gene located on chromosome VI encodes a protein of only 175 amino acids. A frame shift, probably resulting from sequencing errors, was corrected in the *YNG2* ORF (nucleotides 15245 to 16880 with a probable frame shift near 16430) and in the *YFL5* ORF (nucleotides 242032 to 242986 with a probable frame shift near 242182 and a sequence error near 242045).

The codon adaptation index (CAI) values, which may be related to the level of expression of genes (Sharp and Li, 1987), range from 0·090 to 0·538. The CAI value of the gene encoding the *AAC2* carrier, which is the expressed gene in aerobic conditions (Drgon *et al.*, 1992), is higher than that of the *AAC1* and *AAC3* isoforms and higher than that of other yeast carriers, suggesting that the adenylic carrier is the most abundant.

As mentioned in Table 1, the function of only five yeast mitochondrial carriers is known: three isoforms of the ADP/ATP carrier, the phosphate and citrate carriers. Flx1p may be a carrier for the import of FAD as suggested by Tzagoloff (Wu *et al.*, 1995; Tzagoloff *et al.*, 1996).

Sequence alignment and phylogenetic tree

The sequences of the putative carriers of yeast shown in Table 1 and those of four bovine carriers (oxoglutarate carrier, ADP/ATP carrier, uncoupling protein and Grave's disease carrier (GDC)) have been aligned using progressive pairwise alignments. Figure 1 illustrates the phylogenetic tree thus obtained.

It is obvious that six sequences are highly similar to those of some other yeast carriers. Three yeast isoforms exist for the ADP/ATP carrier as shown previously (Adrian *et al.*, 1986; Lawson and Douglas, 1988; Kolarov *et al.*, 1990) and the distances between them are 1·26, 1·28 and 1·4. Sequence comparison between Mrs3p and Mrs4p, between Ymc1p and Ymc2p, between Yea6p and Yia6p, between MPCP and Yeo3p and between YO1 and Lpi11p reveals distances of 1·19, 1·13, 0·97, 0·74 and 1·09 respectively, indicating that the five pairs could be isoforms. Therefore, the 34 putative carriers of yeast can be classified into 27 subfamilies with 27 different functions; the function of only three of them has been identified (adenylic, phosphate and citrate carriers).

Two yeast ORFs present a high identity with bovine carriers: the *YL1* gene could encode the yeast oxoglutarate carrier as the distance between it and the bovine, rat or human oxoglutarate carriers (0·68) is close to that between yeast and bovine citrate or phosphate carriers (0·73 and 0·76 respectively); on the other hand, the *YHG2* product could be a member of the GDC subfamily as the distance between Yhg2p and bovine GDC is equal to 0·68.

Secondary structure and topology prediction

For all the MCF members, the limits of the six putative helices have been defined in the sequence alignment on the basis of the hydrophobicity profiles and in order to minimize the number of charged residues in the helix segments. The odd helices are generally more hydrophobic than the even helices. They are 18 residues long, with a conserved Pro in position 17 and are often followed by a sequence containing the mitochondrial energy transfer protein signature. The even helices are 19 residues long, have a Gly as first residue and are stabilized by aromatic residues (Phe, Tyr and Trp), which are concentrated in the membrane domain close to the lipid head-group region. For all the putative yeast carriers and all the known carriers in higher eukaryotes, the matricial

Table 1. Putative members of the yeast mitochondrial carrier family with abbreviations used, names in YPD and MIPS, accession numb and GenBank, the chromosomal localization of the 34 ORFs, the number of amino acid residues of the proteins, the codon adaptation index the function when known.

Name	YPD	MIPS	PIR	GenBank	Chromosome	Residues	CAI	Function	References
AAC2	AAC2	YBL030c	A31978	Z35791x1	II	318	0·54	ADP/ATP	Lawson and Douglas (1988)
AAC3	AAC3	YBL045w	A36582	Z35954x1	II	307	0·20	ADP/ATP	Kolarov *et al.* (1990)
RIM2	RIM2	YBL192w	S36081	Z36061x1	II	377	0·12		Demolis *et al.* (1993); Van Dyck *et al.*
YB8E	YBR291C	YBR291c	S44554	Z36160x1	II	299	0·15	Citrate	Kaplan *et al.* (1995)
YMC2	YMC2	YBL104w	S48269	Z35973x1	II	329	0·12		Mannhaupt *et al.* (1994)
SHM1	SHM1	YDL198c	S48537	X83276x3	IV	300	0·21		McNeil *et al.* (1994)
YD1	YDL119c	YDL119c	S67662	Z74167x1	IV	307	0·09		
YEA6	YEL006w	YEL006w	S50453	U18530x26	V	335	0·11		
YEO3	YER053c	YER053c	S50556	U18796x21	V	300	0·16		
YFL5	YFR045w	YFR045w	S56300	D50617x115	VI	318*	0·10		
YG1	YGR096w	YGR096w	S64401	Z72881x1	VII	314	0·12		
YG2	YGR257c	YGR257c	S64589	Z73042x1	VII	366	0·10		
YHG2	YHR002w	YHR002w	S46795	U10555x6	VIII	357	0·10		
YIA6	YIL006w	YIL006w	S48451	Z47047x172	IX	373	0·10		
YIN4	FLX1	YIL134w	S48400	Z47047x44	IX	311	0·11	FAD?	Wu *et al.* (1995); Tzagoloff *et al.* (1996
ACR1	ACR1	YJR095w	S43280	Z49595x1	X	322	0·21		Fernández *et al.* (1994)
MPCP	MIR1	YJR077w	S12318	Z49577x1	X	311	0·36	Phosphate	Phelps *et al.* (1991)
MRS3	MRS3	YJL133w	S55179	Z49408x1	X	314	0·08		Wiesenberger *et al.* (1991); Schmidt *et*
MRS4	MRS4	YKR052c	S13533	Z28277x1	XI	304	0·12		Wiesenberger *et al.* (1991)
PMT	YKL120W	YKL120w	S25357	Z28120x1	XI	324	0·19		Colleaux *et al.* (1992)
YL1	YLR348c	YLR348c	S51351	U19028x14	XII	298	0·13		
AAC1	AAC1	YMR056c	A24849	Z49703x9	XIII	309	0·11	ADP/ATP	Adrian *et al.* (1986)
YM1	YMR166c	YMR166c	S54524	Z49705x15	XIII	368	0·11		
YM2	YMR241w	YMR241w	S56055	Z48756x3	XIII	314	0·33		
PET8	PET8	YNL003c	S45458	X77114x3	XIV	284	0·12		Verhasselt *et al.* (1994); Lalo *et al.* (19
YNG2	YNL083w	YNL083w	S57539	X89016x7	XIV	545*	0·13		
ARG11	ARG11	YOR130c	S60997	X90518x20	XV	292	0·10		Crabeel *et al.* (1996); Wieman *et al.* (1
YO1	YOR222w	YOR222w	S60949	X92441x12	XV	307	0·18		
YO2	YOR100c	YOR100c	S61660	X94335x19	XV	327	0·13		
YMC1	YMC1	YPR058w	S54080	Z49219x14	XVI	307	0·15		Graf *et al.* (1993)
YP1	YPR021c	YPR021c	S54495	Z49274x1	XVI	902	0·14		
YP2	YPR011c	YPR011c	S57544	Z49919x4	XVI	326	0·10		
YP3	YPR128c	YPR128c	S69019	U40829x1	XVI	328	0·14		
LPI11	YPL134c	YPL134c	S69050	U43703x11	XVI	310	0·12		

*Number of residues after frame shift correction.

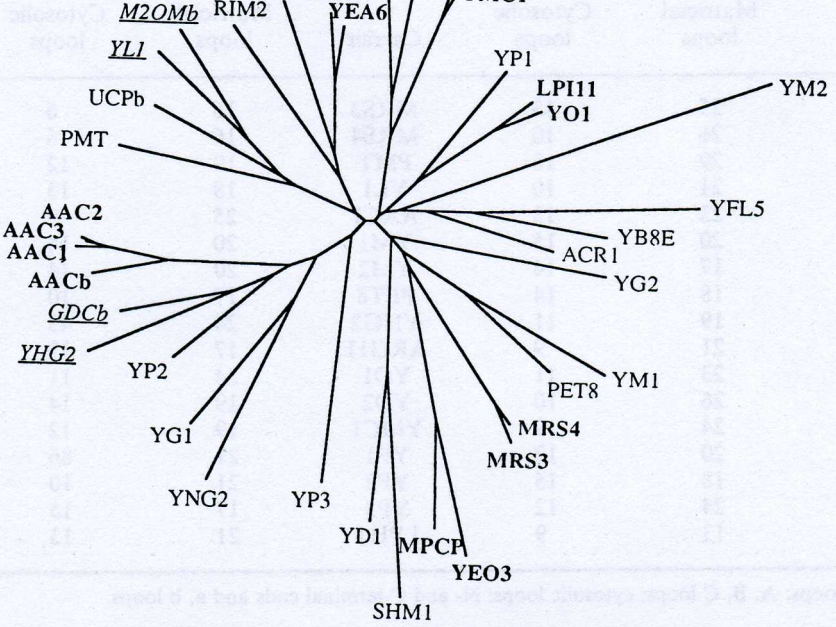

Figure 1. Phylogenetic tree of the putative members of the mitochondrial carrier family. The yeast sequences are as indicated in Table 1. M2OMb, bovine oxoglutarate carrier (A36305); UCPb, bovine uncoupling protein (P10861); AAC2b, bovine ADP/ATP carrier, isoform 2 (P32007); GDCb, bovine Grave's disease carrier (S25596). Isoforms are in bold and italics.

hydrophilic loops are longer than the cytosolic loops.

As the previously proposed signature is not detected (three sequences) or is detected only once (nine sequences), it has to be modified to better characterize the yeast MCF. The new motif: P-φ-(DE)-(φT)-(φT)-(RK)-(Tφ)-(RK)-φ (φ=hydrophobic residue), which is found two or three times in all sequences except YP3 and YIN4 (in which it appears only once), can be proposed as the mitochondrial carrier signature.

The strategy proposed by Von Heijne (1994) to predict topology has been applied to the mitochondrial carriers. This approach, called the 'positive inside rule', is complementary to the standard hydrophobicity analysis and predicts the orientation of the transmembrane proteins on the basis of the bias in the distribution of Arg+Lys residues in the hydrophilic loops (<60 residues) on both sides of the membrane. The six-helix model with both extremities exposed at the cytoplasmic side, leads to a number of Arg+Lys residues that is always higher on the matricial side (Table 2). This approach cannot be applied to the YP1 and YNG2 carriers, which have an N-terminal end of 533 and 230 amino acid residues respectively. The distribution of charges shown in Table 2 is in agreement with the 'positive inside rule', suggesting that the membrane insertion of all mitochondrial carriers must be similar. These topological predictions based on the sequence analysis have been partially confirmed experimentally in higher eukaryotes for several carriers of known function (ADP/ATP, citrate, oxoglutarate and phosphate carriers and uncoupling protein; Ferreira *et al.*, 1990; Capobianco *et al.*, 1991, 1995; Brandolin *et al.*, 1989; Bisaccia *et al.*, 1994; Marty *et al.*, 1992; Eckerskorn and Klingenberg, 1987; Miroux *et al.*, 1992, 1993).

Main features of the functional subfamilies

It is already known that the four charged residues located in the membrane segments of the

Carrier	Matricial loops	Cytosolic loops	Carrier	Matricial loops	Cytosolic loops
AAC2	25	13	MRS3	15	6
AAC3	26	10	MRS4	16	6
RIM2	29	18	PMT	19	12
YB8E	21	10	YL1	18	13
YMC2	23	13	AAC1	25	9
SHM1	20	15	YM1	20	14
YD1	17	14	YM2	20	14
YEA6	18	14	PET8	17	10
YEO3	19	11	YNG2	24	43
YFL5	21	9	ARG11	17	12
YG1	23	11	YO1	24	11
YG2	26	10	YO2	19	14
YHG2	24	19	YMC1	19	12
YIA6	20	12	YP1	21	86
YIN4	18	15	YP2	21	10
ACR1	24	12	YP3	17	15
MPCP	13	9	LPI11	21	13

Matricial loops: A, B, C loops; cytosolic loops: N- and C-terminal ends and a, b loops.

ADP/ATP carrier are absolutely conserved and that a single mutation of one of them leads to a failure to grow on a non-fermentable carbon source such as glycerol. These four residues are essential for the protein function (Nelson *et al.*, 1993). The number and the position of the charged residues in the helices of the members of MCF mentioned in Figure 1 have been analysed. Table 3 shows that, for all the functionally known carriers, the charged residues are well conserved and characteristic of each subfamily. These carriers (ADP/ATP, citrate, oxoglutarate, phosphate and UCP) have a net positive charge in the hydrophobic domain that could be correlated with the anionic nature of the substrate. Indeed, it has been shown recently that the uncoupling protein does not transport protons but fatty acid anions and that fatty acids induce proton transport because they can diffuse electroneutrally across the membrane (Garlid *et al.*, 1996). Analysis of the helices of the yeast isoforms also indicates a strict conservation of the number and the position of charged residues in each subfamily, suggesting a functional role for these residues. These isoforms also have a net positive charge, except Mrs3p and Mrs4p, which have a null net charge.

The classification into subfamilies according to the conserved charged residues in the helices is in agreement with that resulting from the phylogenetic tree. Both approaches give evidence of the existence of a total of 27 functionally distinct carriers in yeast mitochondria.

The comparison of the hydrophilic segments indicates a low sequence isology within each subfamily. External loop residues are not conserved while in the internal loops, the N-terminal signature and some charged residues at their C-terminal end are conserved. Moreover a very striking peculiarity of all AAC carriers is the presence of the highly conserved RRRMMM motif at the beginning of the N-terminal end of the matricial C loop (Nelson *et al.*, 1993).

CONCLUSIONS

The present analysis shows the usefulness of phylogenetic analysis when applied to the data provided by the systematic sequencing of a genome. It has allowed us to demonstrate the existence of 27 functionally distinct mitochondrial carriers within the whole yeast genome. The function of three carriers is already known and putative yeast oxoglutarate carriers and a protein analogous to

Carrier	Helix I	Helix II	Helix III	Helix IV	Helix V	Helix VI	Charges (+)
AACh/A29135	K	R		R		R	4
AAC1	K	R		R		R	4
AAC2	K	R		R		R	4
AAC3	K	R		R		R	4
UCPh/A60793	D	R R		R E		R	2
UCPr/A26294	D	R R		R E		R	2
UCPb/P10861	D	R R		E		R	1
MPCPr/A34350		K	D E	K R		R	2
MPCPb/P12234		K	D E	K R		R	2
MPCP		K	D E	K K		R	2
YEO3		K	D E	K R		R	2
M2OMb/A36305		R R		R		R	4
M2OMh/S29598		R R		R		R	4
YL1		R R		K R		R	5
CITr/A46596	E	K R	E	K R		R R D	3
YB8E	E	K R	E	K R		R R	4
MRS3	E		D			K R	0
MRS4	E		D			K R	0
YMC1				R		R	2
YMC2				R		R	2
YIA6					K	R	2
YEA6					K	R	2
YO1	E	K K R E	E	R E		K R	2
LPI11	E	K K R E	E	R E		K R	2
GDCb/S26596	K	R				R	3
YHG2	K	R				K	3
RIM2		R		E	K	R	2
YIN4					K E	R	1
ACR1	E	K R	E	R		R R	3
PMT		R		R D		K R	3
PET8	D		R E	R E		R	0
SHM1	E	K K R	E	R		K K	4
ARG11	K E	E		R E		R	0
YM1	K D		D	K R D		R	0
YM2	E	K E		R R		R	2
YP1		K K E		R D		R R	3
YP2	R	R				R	3
YP3				R	K	K	3
YO2				R D	D	R	0
YNG2	R	K K E				K	3
YG2			R	R E D		R R	2
YD1		R	R	R D		R R K	5
YG1	R				K	K	3

AAC, ADP/ATP carrier; UCP, uncoupling protein; M2OM, oxoglutarate carrier; MPCP, phosphate carrier; CIT, citrate carrier from beef (b), human (h) and rat (r).

the GDC have been identified by sequence isology with bovine carriers. Obviously, most of the 22 other subfamilies have to catalyse the transport of already known substrates but, given their number, the existence of carriers whose function has not yet been identified is highly probable.

Finally, the previously proposed mitochondrial energy transfer protein signature has been revised

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