Carnitine in yeast and filamentous fungi

by

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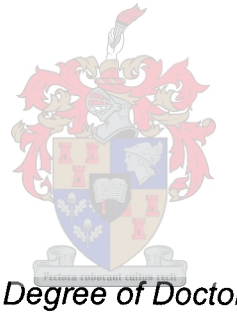

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

In the yeast *Saccharomyces cerevisiae*, two biochemical pathways ensure that activated cytoplasmic or peroxisomal acetyl-groups are made available for mitochondrial energy production when the cells utilise non-fermentable carbon sources. The first pathway is the glyoxylate cycle, where two activated acetyl-groups are incorporated into each cycle, which releases a C_4 intermediate. This intermediate is then transported to the mitochondria where it can enter the tricarboxylic acid cycle. The second pathway is the carnitine shuttle. Activated acetyl-groups react with carnitine to form acetylcarnitine, which is then transported to the mitochondria where the acetyl group is transferred.

In this study it was shown that the deletion of the glyoxylate cycle specific citrate synthase, encoded by *CIT2*, results in a strain that is dependent on carnitine for growth on non-fermentable carbon sources. Using a Δ*cit2* strain, mutants affected in carnitine-dependent metabolic activities were generated. Complementation of the mutants with a genomic library resulted in the identification of four genes involved in the carnitine shuttle. These include: (i) the mitochondrial and peroxisomal carnitine acetyltransferase, encoded by *CAT2*; (ii) the outer-mitochondrial carnitine acetyltransferase, encoded by *YAT1*; (iii) the mitochondrial carnitine translocase, encoded by *CRC1*; and (iv) a newly identified carnitine acetyltransferase, encoded by *YAT2*. All three carnitine acetyltransferases are essential in a carnitine-dependent strain.

The dependence on exogenous carnitine of the Δ*cit2* strain when grown on non-fermentable carbon sources suggested that *S. cerevisiae* does not biosynthesise carnitine. Measurements using electrospray mass spectrometry confirmed this hypothesis. As a result an investigation was initiated into carnitine biosynthesis in order to genetically engineer a *S. cerevisiae* strain that could endogenously biosynthesise carnitine.

The filamentous fungus, *Neurospora crassa*, was one of the first organisms used in the seventies to identify the precursor and intermediates of carnitine biosynthesis. However, it was only about twenty years later that the first genes encoding these enzymes where characterised. Carnitine biosynthesis is a four-step process, which starts with trimethyllysine as precursor. Trimethyllysine is converted to hydroxy-trimethyllysine by the enzyme trimethyllysine hydroxylase (TMLH). Hydroxy-trimethyllysine is cleaved to trimethylamino-butyraldehyde by the hydroxytrimethyllysine aldolase (HTMLA) releasing glycine. Trimethylamino-butyraldehyde is dehydrogenated to trimethylamino-butyrate (γ-butyrobetaine) by trimethylamino-butyraldehyde dehydrogenase (TMABA-DH). In the last step, γ-butyrobetaine is converted to L-carnitine by γ-butyrobetaine hydroxylase (BBH).

The *N. crassa* TMLH homologue was identified in the genome database based on the protein sequence homology of the human TMLH. Due to the high amount of

introns predicted for this gene, the cDNA was cloned and subjected to sequencing, which then revealed that the gene indeed had seven introns. Functional expression of the gene in *S. cerevisiae* and subsequent enzymatic analysis revealed that the gene coded for a TMLH. It was therefore named *cbs-1* for "carnitine biosynthesis gene no. 1". Most of the kinetic parameters were similar to that of the human TMLH enzyme. Following this, a genomic copy of the *N. crassa* BBH homologue was cloned and functionally expressed in *S. cerevisiae*. Biochemical analysis revealed that the BBH enzyme could biosynthesise L-carnitine from γ-butyrobetaine and the gene was named *cbs-2*. In addition, the gene could rescue the growth defect of the carnitine-dependent Δ*cit2* strain on non-fermentable carbon sources when γ-butyrobetaine was present. This is the first report of an endogenously carnitine biosynthesising strain of *S. cerevisiae*.

The cloning of the remaining two biosynthesis genes presents particular challenges. To date, the HTMLA has not been characterised on the molecular level making the homology-based identification of this protein in *N. crassa* impossible. Although the TMABA-DH has been characterised molecularly, the protein sequence is conserved for its function as a dehydrogenase and not conserved for its function in carnitine biosynthesis, as in the case of TMLH and BBH. The reason for this is probably due to the fact that the enzyme is involved in other metabolic processes. The use of *N. crassa* carnitine biosynthesis mutants would probably be one way in which to overcome these obstacles.

The Δ*cit2* mutant proved useful in studying carnitine related metabolism. We therefore searched for suppressors of Δ*cit2*, which resulted in the cloning of *RAS2*. In *S. cerevisiae*, two genes encode Ras proteins, *RAS1* and *RAS2*. GTP-bound Ras proteins activate adenylate cyclase, Cyr1p, which results in elevated cAMP levels. The cAMP molecules bind to the regulatory subunit of the cAMP-dependent kinase (PKA), Bcy1p, thereby releasing the catalytic subunits Tpk1p, Tpk2p and Tpk3p. The catalytic subunits phosphorylate a variety of regulators and enzymes involved in metabolism. Overexpression of *RAS2* could suppress the growth defect of the Δ*cit2* mutant on glycerol. In general, overexpression of *RAS2* enhanced the proliferation of wild-type cells grown on glycerol. However, the enhancement of proliferation was much better for the Δ*cit2* strain grown on glycerol. In this respect, the retrograde response may play a role. Overexpression of *RAS2* resulted in elevated levels of intracellular citrate and citrate synthase activity. It therefore appears that the suppression of Δ*cit2* by *RAS2* overexpression is a result of the general upregulation of the respiratory capacity and possible leakage of citrate and/or citrate synthase from the mitochondria. The phenotype of *RAS2* overexpression contrasts with the hyperactive $RAS2^{val19}$ allele, which causes a growth defect on glycerol. However, both *RAS2* overexpression and $RAS2^{val19}$ activate the cAMP/PKA pathway, but the $RAS2^{val19}$ dependent activation is more severe. Finally, this study implicated the Ras/cAMP/PKA pathway in the proliferation effect on glycerol by showing that in a Δ*tpk1* strain, the growth effect is blocked. However, the enhanced proliferation was

still observed in the Δ*tpk2* and Δ*tpk3* strains when *RAS2* was overexpressed. Therefore, it seems that Tpk1p plays an important role in growth on non-fermentable carbon sources, a notion that is supported by the literature.

OPSOMMING

In die gis *Saccharomyces cerevisiae*, is daar twee metaboliese weë waarmee geaktiveerde asetielgroepe na die mitochondrium vervoer kan word wanneer die sel op nie-fermenteerbare koolstofbronne groei. Die een weg is die glioksilaatsiklus, waar die geaktiveerde asetielgroepe geïnkorporeer word in die siklus en dan vrygestel word as C_4-intermediêre. Hierdie intermediêre word dan na die mitochondrium vervoer waar dit in die trikarboksielsuursiklus geïnkorporeer word. Die ander weg is die karnitiensiklus, waar geaktiveerde asetielgroepe met karnitien reageer om asetielkarnitien te vorm wat dan na die mitochondrium vervoer word waar dit die asetielgroep weer vrygestel.

Hierdie studie het getoon dat die delesie van die glioksilaatsiklus spesifieke sitraatsintetase, gekodeer deur *CIT2*, die gisras afhanklik maak van karnitien vir groei op nie-fermenteerbare koolstofbronne. Deur gebruik te maak van 'n Δ*cit2* gisras, kon mutante, wat geaffekteer is in karnitien-verwante metaboliese aktiwiteite, gegenereer word. Komplementering van die mutante met 'n genomiese biblioteek het gelei tot die identifisering van vier gene betrokke by die karnitiensiklus. Hierdie gene sluit in: (i) die mitochondriale en die peroksisomale karnitienasetieltransferase, gekodeer deur *CAT2*; (ii) die buite-mitochondriale karnitienasetieltransferase, gekodeer deur *YAT1*; (iii) die mitochondriale karnitientranslokase, gekodeer deur *CRC1*; en (iv) 'n nuut-geïdentifiseerde karnitienasetieltransferase, gekodeer deur *YAT2*. Daar benewens, is ook gewys dat al drie karnitienasetieltransferases noodsaaklik is in 'n karnitien-afhanklike gisras.

Die afhanklikheid van eksogene karnitien van die Δ*cit2* gisras, wanneer dit gegroei word op nie-fermenteerbare koolstofbronne, was aanduidend dat *S. cerevisiae* nie karnitien kan biosintetiseer nie. Metings deur middel van elektronsproeimassaspektrometrie het hierdie veronderstelling bevestig. Gevolglik is 'n ondersoek deur ons geïnisieer in die veld van karnitienbiosintese om 'n *S. cerevisiae* gisras geneties te manipuleer om karnitien sodoende endogenies te biosintetiseer.

Die filamentagtige fungus, *Neurospora crassa*, was een van die eerste organismes wat in die sewentiger jare gebruik is om die voorloper en intermediêre van karnitienbiosintese te identifiseer. Dit was egter eers sowat twintig jaar later dat die eerste gene wat vir hierdie ensieme kodeer, gekarakteriseer is. Karnitienbiosintese is 'n vierstap-proses wat met trimetiellisien as voorloper begin. Trimetiellisien word omgeskakel na hidroksi-trimetiellisien deur die ensiem trimetiellisienhidroksilase (TMLH). Hidroksietrimetiellisien word dan gesplits om trimetielaminobuteraldehied te vorm deur die werking van die hidroksitrimetiellisienaldolase (HTMLA) met die gevolglike vrystelling van glisien. Trimetielaminobuteraldehied word dan na trimetielaminobuteraat (γ-butirobeteïen) deur trimetielaminobuteraldehied dehidrogenase (TMABA-DH) gedehidrogeneer. In

die laaste stap word γ-butirobeteïen deur middel van die γ-butirobeteïen hidroksilase (BBH) na L-karnitien omgeskakel.

Op grond van die proteïenvolgordehomologie in die genoomdatabasis tussen die menslike TMLH en *N. crassa* se TMLH is laasgenoemde geïdentifiseer. As gevolg van die groot getal introns wat vir hierdie geen voorspel is, is die cDNA-weergawe daarvan gekloneer en aan volgordebepaling onderwerp. Dit het getoon dat die geen inderdaad sewe introns bevat. Funksionele uitdrukking van die geen in *S. cerevisiae* en ensiematiese analise het getoon dat die geen vir 'n TMLH kodeer en is gevolglik *cbs-1* genoem; dit staan vir "karnitien biosintese geen no. 1". Meeste van die kinetiese parameters was ook soortgelyk aan die van die menslike TMLH-ensiem. Hierna is 'n genomiese kopie van *N. crassa* se BBH-homoloog gekloneer en funksioneel in *S. cerevisiae* uitgedruk. Biochemiese analise het getoon dat die uitgedrukte BBH-ensiem L-karnitien vanaf γ-butirobeteïen kan biosintetiseer en die geen is *cbs-2* genoem. Daar benewens kon die geen die groeidefek van die karnitien-afhanklike Δ*cit2*-gisras ophef wanneer dit op nie-fermenteerbare koolstofbronne in die teenwoordigheid van γ-butirobeteïen aangekweek is. Hierdie is die eerste verslag oor 'n endogeniese karnitien-biosintetiserende ras van *S. cerevisiae*.

Die klonering van die oorblywende twee karnitienbiosintetiserende gene het sekere uitdagings. Tot op datum, is die HTMLA nog nie tot op genetiese vlak gekarakteriseer nie, wat dan die homologie-gebaseerde identifikasie van hierdie proteïen in *N. crassa* onmoontlik maak. Alhoewel die TMABA-DH geneties gekarakteriseer is, is die proteïenvolgorde ten opsigte van sy funksie as 'n dehidrogenase gekonserveer, maar nie vir sy funksie in karnitienbiosintese soos in die geval van TMLH en BBH nie. Die rede hiervoor is moontlik omdat die ensiem ook in ander metaboliese prosesse betrokke is. Die gebruik van *N. crassa* karnitienmutante sal moontlik een manier wees om hierdie probleme te oorkom.

Die Δ*cit2*-mutant het handig te pas gekom vir die bestudering van karnitien-verwante metabolisme. Dus is daar vir onderdrukkers van die Δ*cit2*-mutant gesoek wat gelei het tot die klonering van die *RAS2*-geen. In *S. cerevisiae*, kodeer twee gene vir Ras-proteïene, *RAS1* en *RAS2*. GTP-gebonde Ras-proteïene aktiveer adenilaatsiklase, Cyr1p, wat verhoogde intrasellulêre cAMP-vlakke tot gevolg het. Die cAMP bind aan die regulatoriese subeenheid van die cAMP-proteïenkinase (PKA), Bcy1p, en daardeur word die katalitiese subeenhede, Tpk1p, Tpk2p en Tpk3p, vrygestel. Die katalitiese subeenheid fosforileer 'n verskeidenheid van reguleerders en ensieme betrokke by metabolisme. Ooruitdrukking van *RAS2* het die groeidefek van die Δ*cit2*-mutant op gliserol onderdruk. Oor die algemeen, verbeter die ooruitdrukking van *RAS2* die proliferasie van die wildetipe op gliserol bevattende media. Alhoewel, die verbetering van proliferasie was baie meer opmerklik in die Δ*cit2*-gisras. In hierdie verband, speel die gedegenereerde response dalk 'n rol. Ooruitdrukking van *RAS2* het verhoogde intrasellulêre vlakke van sitraat- en sitraatsintetase-aktiwiteit tot gevolg gehad. Dit wou dus voorkom asof die

onderdrukking van die Δ*cit2*-groeidefek deur *RAS2* se ooruitdrukking die gevolg was van algemene opregulering van respiratoriese kapasiteit en die lekkasie van sitraat en/of sitraatsintetase uit die mitochondria. Die fenotipe van *RAS2* ooruitdrukking kontrasteer die hiperaktiewe $RAS2^{val19}$ alleel, wat 'n groeidefek op gliserol media veroorsaak. Alhoewel beide *RAS2*-ooruitdrukking en $RAS2^{val19}$ die cAMP/PKA-weg aktiveer, is gevind dat die $RAS2^{val19}$-afhanklike aktivering strenger is. Ten slotte, die cAMP/PKA-weg is in die proliferasie effek op gliserol media geïmpliseer deur te wys dat in 'n Δ*tpk1*-gisras, die groeieffek geblokkeer is. Alhoewel, die verbeterde proliferasie is steeds waargeneem in die Δ*tpk2*-en Δ*tpk3*-gisrasse toe die *RAS2*-geen ooruitgedruk is. Dus, dit wil voorkom asof Tpk1p 'n belangrike rol in die groei van gisselle op nie-fermenteerbare koolstofbronne speel; 'n veronderstelling wat deur die literatuur ondersteun word.

This dissertation is dedicated to my late mother-in-law, Lis Joubert
Hierdie proefskrif word aan my ontslape skoonma, Lis Joubert, opgedra

BIOGRAPHICAL SKETCH

Hentie Swiegers was born in Pretoria, South Africa, on the 11th of April 1975. He matriculated at Menlo Park High School, Pretoria, in 1993 and enrolled for a BSc degree at the University of Pretoria in 1994. He obtained a BSc degree (*cum laude*) in Biochemistry and Microbiology in 1996. He obtained a BScHons in Microbiology in 1997 and a MSc (*cum laude*) in Microbiology in 2000 at Stellenbosch University. He enrolled for his PhD degree in Microbiology in 2000.

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PREFACE

This dissertation is presented as a compilation of seven chapters including an appendix. With the exception of the first, second and the last chapters (Chapter 1, Chapter 2 and Chapter 7), this dissertation is a collection of manuscripts that were published or will be submitted for publication in different international scientific journals. In order to maintain stylistic continuity, all text and figures were formatted to the same style.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **LITERATURE REVIEW**
The metabolic function of carnitine acetyltransferase and carnitine biosynthesis enzymes

RESEARCH RESULTS

Chapter 3 Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: Three carnitine acetyltransferases are essential in a carnitine-dependent strain (published in *YEAST*).

Chapter 4 Carnitine biosynthesis in *Neurospora crassa*: Identification of a cDNA coding for ε-*N*-trimethyllysine hydroxylase and its functional expression in *Saccharomyces cerevisiae* (published in *FEMS Microbiology Letters*).

Chapter 5 Engineering carnitine biosynthesis in *Saccharomyces cerevisiae*: functional expression of a γ-butyrobetaine hydroxylase from *Neurospora crassa* (will be submitted to *Biotechnology and Bioengineering*).

Chapter 6 Regulation of respiratory growth by Ras: The glyoxylate cycle mutant, Δ*cit2*, is suppressed by *RAS2* (will be submitted to *Journal of Biological Chemistry*).

Chapter 7 **General Discussion and Conclusions**

Appendix The determination of carnitine acetyltransferase activity in *Saccharomyces cerevisiae* by HPLC-Electrospray Mass Spectrometry (submitted to *Analytical Biochemistry*)

CONTENTS

CHAPTER 1

General introduction and project aims

1. Introduction

The yeast *Saccharomyces cerevisiae* has for more than a hundred years contributed enormously to our understanding of biochemical pathways. Since Louis Pasteur first proposed in 1860 that living cells, or rather yeast, convert grape sugars to alcohol, this organism has been used as a model system. Later, Hans and Eduard Buchner showed in 1897 that cell free extracts of yeast can convert sugar into ethanol and thereby started to elucidate the metabolic pathway of glycolysis. This work initiated the field of metabolic pathway analysis in living systems (Cohen, 1984). Today, with the advent of yeast genetics, the genetic components of most biochemical pathways have been determined, contributing to the molecular understanding of metabolism.

A biochemical pathway that has long been known, but has not been investigated in yeast in any detail, is referred to as the carnitine shuttle. However, a significant amount of data have been generated with regards to the function and genetic components of the carnitine shuttle in mammalian cells.

2. Metabolic role of carnitine

In mammalian cells, the carnitine shuttle serves an essential function by transferring activated short- and long-chained fatty acids into the mitochondria and the shuttle is therefore required for survival. Carnitine reacts with acyl-CoA, to form acylcarnitine and free CoA in the cytoplasma through the action of carnitine acyltransferases. Acylcarnitine can then be translocated across the inner-mitochondrial membrane through the carnitine-acylcarnitine translocase. Inside the mitochondria, the acyl group is released to form acyl-CoA and carnitine; and the carnitine is shuttled back to the cytoplasma. In this way, there can be a continuous flow of short- and long-chained fatty acids into the mitochondria (Bieber, 1988).

Unlike mammalian cells, yeast does not generate and use long-chain acyl-CoA groups; and only the 2-carbon acetyl-CoA moiety is present. Subsequently, carnitine acetyltransferases (CATs) are the only carnitine acyltransferases present in yeast (Bremer, 1983). Kispal et al. (1993) cloned the first CAT from yeast using specific CAT antibodies that allowed them to identify the cDNA clones. They showed that this CAT, encoded by *CAT2*, is responsible for most of the measured enzyme activity in yeast. At the same time another CAT, encoded by *YAT1*, was cloned and shown to be associated with the outer-mitochondrial membrane (Schmalix and Bandlow, 1993). These studies were restricted to enzymatic analysis and no significant insights were achieved regarding the metabolic role of the carnitine shuttle. Later, important data were generated with regards to the function of Cat2p in the cell by elucidating the dual location in the peroxisome and the mitochondria when cells were grown on fatty acids (Elgersma et al., 1995). At the same time, in a major breakthrough in the field, it was shown that there are two pathways through which the activated acetyl

groups of acetyl-CoA could enter the mitochondria when cells were grown on fatty acids such as oleate (van Roermund et al., 1995). In these conditions, the fatty acids are broken down in the peroxisome, which is the sole site for fatty acid degradation, to form acetyl-CoA (Kunau et al., 1988). Metabolically, the activated acetyl groups in the peroxisome can be transferred in two ways to the mitochondria for energy production: (i) transfer of the acetyl group to carnitine, through the activity of Cat2p, to form acetylcarnitine, which can then be transferred to the mitochondria or; (ii) entry into the peroxisomal glyoxylate cycle, resulting in the net release of C_4 intermediates, which can be transferred to the mitochondria to enter the tricarboxylic acid (TCA) cycle.

3. Carnitine biosynthesis

Humans, rats, plants and some fungi are able to biosynthesise carnitine from ε-*N*-trimethyllysine (TML) in a four-step process (Lindstedt and Lindstedt, 1970; Kaufman and Broquist, 1977; Bremer, 1983). TML is provided by the lysosomal hydrolysis of proteins that contain this amino acid as a result of the post-translational modification of lysine residues. In the first step of the carnitine biosynthesis, TML is hydroxylated to β-hydroxy-ε-*N*-trimethyllysine by ε-*N*-trimethyllysine hydroxylase (Bremer, 1983; Rebouche and Engel, 1980). Subsequently, β-hydroxy-ε-*N*-trimethyllysine, is cleaved into γ-trimethyl-aminobutyraldehyde and glycine by β-hydroxy-ε-*N*-trimethyllysine aldolase (Bremer, 1983; Rebouche and Engel, 1980). The aldehyde is then oxidised by γ-trimethyl-aminobutyraldehyde dehydrogenase to form γ-butyrobetaine (Hulse and Henderson, 1980; Rebouche and Engel,1980; Bremer, 1983). Finally, γ-butyrobetaine is hydroxylated at the 3-position by γ-butyrobetaine hydroxylase to form L-carnitine (Englard, 1979; Bremer, 1983; Rebouche and Engel, 1980).

The genetic code for one of these enzymes was first elucidated in 1998 (Vaz et al., 1998). Subsequently, enzymes required for the catalysis of three of the four reactions required for carnitine biosynthesis have been characterised at the molecular level, either in rat, mouse or man, most of the work being done by the same group (Vaz et al., 1998; Galland et al., 1999, Vaz et al., 2000; Vaz et al., 2001).

4. Project background

The initial aim of our research was to clone a carnitine transporter from *S. cerevisiae*. It was decided to use a mutant screen approach and the question arose whether the carnitine shuttle is the only pathway through which activated acetyl groups can be transferred to the mitochondria. The answer to this question was experimentally demonstrated by showing that by deleting the glyoxylate cycle citrate synthase, encoded by *CIT2*, the cells grew normally on fatty acids but they did not grow in

these conditions when the carnitine acetyltransferase, encoded by *CAT2*, was also deleted. Deletion of *CAT2* alone did not affect growth on fatty acids. Therefore, in the Δ*cit2* mutant strain, the cell is dependent on the carnitine shuttle, and subsequently L-carnitine, to grow in these conditions (van Roermund et al., 1995).

The glyoxylate cycle is a modified TCA cycle, which incorporates acetyl-CoA, but instead of releasing CO_2, releases succinate, requiring the inflow of two acetyl-CoAs and the unique intermediate, glyoxylate (Kornberg, 1966). As in the case of the TCA cycle, the incorporation of acetyl-CoA is catalysed by a citrate synthase, Cit2p, through the binding of acetyl-CoA to oxaloacetate, releasing CoA and forming citrate. The glyoxylate cycle citrate synthase, Cit2p, is cytosolic (and peroxisomal when cells are grown on fatty acids) whereas the TCA cycle citrate synthase, Cit1p, is mitochondrial (Kispal et al., 1988). Another mitochondrial citrate synthase exists, Cit3p, but its specific function is not clear (Jia et al., 1997). Deleting either Cit1p or Cit2p does not result in a phenotype on rich media with a non-fermentable carbon source, but deleting both results in a severe growth defect in these conditions (Kispal et al., 1988).

The phenotype of the Δ*cit2* mutant grown on non-fermentable carbon sources would indicate to us if *S. cerevisiae* could endogenously biosynthesise carnitine. By growing the Δ*cit2* strain on synthetic non-fermentable carbon sources (no carnitine), growth would indicate endogenous biosynthesis and no growth no biosynthesis. Results obtained showed that the latter is the case and that exogenous carnitine could rescue this growth defect. Therefore, a carnitine-dependent strain of *S. cerevisiae* was generated. This strain allowed; (i) the development of a genetic screen to identify genes coding for proteins involved in the carnitine shuttle; (ii) the assesment of the ability of a heterologous gene product to endogenously produce carnitine in *S. cerevisiae*; (iii) the development of a plate assay for the identification of carnitine producing microorganisms; and (iv) the cloning of carnitine-independent suppressors of the Δ*cit2* mutant. Indeed, this strain was used in all four papers presented in the results section of this work (Chapters 3-6).

5. Specific aims

The specific aims of this study were:

(i) to identify genes encoding proteins involved in the carnitine shuttle;
(ii) to assert the specific function of the proteins in the carnitine shuttle;
(iii) to heterologously clone and express possible carnitine biosynthesising genes;
(iv) to characterise the cloned genes and products on genetic and enzymatic level;
(v) to develop a carnitine biosynthesising *S. cerevisiae* strain; and

(vi) to develop a better understanding of the regulation and metabolism of *S. cerevisiae* on non-fermentable carbon sources in regards to the carnitine shuttle and glyoxylate cycle.

The results and discussions regarding the fulfilment of these aims are discussed in Chapters 3-6. To give a comprehensive background to the work, a literature review, Chapter 2, was compiled on the role of carnitine acyltransferase and carnitine biosynthesis enzymes in eukaryotic metabolism.

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CHAPTER 2

Literature review

The metabolic function of carnitine acyltransferase and carnitine biosynthesis enzymes

1. The carnitine acyltransferase system

1.1 Carnitine: The molecule and its history

Carnitine (L-3-hydroxy-4-*N,N,N*-trimethylaminobutyrate) is a quaternary ammonium compound with high polarity. It was discovered more than a century ago in muscle cells and its chemical structure was determined 30 years later (Tomita and Sendju, 1927; Bremer, 1983). The metabolic importance of carnitine came to light during the middle of the last century when it was shown that carnitine is essential for the mealworm *Tenebrio molitor*, hence the other name, vitamin B_T (Carter et al., 1952). Later, the role of carnitine in the shuttling of acyl groups to the mitochondria was discovered by two sets of work, which indicated that carnitine could be reversibly acetylated by acetyl-CoA, and that carnitine induced β-oxidation (Friedman and Fraenkel, 1955; Fritz 1963). Today, a literature search using carnitine as keyword delivers more than 7000 scientific publications in peer-reviewed journals, indicating its importance in living systems. The chemical structure of carnitine is shown in Figure 1.

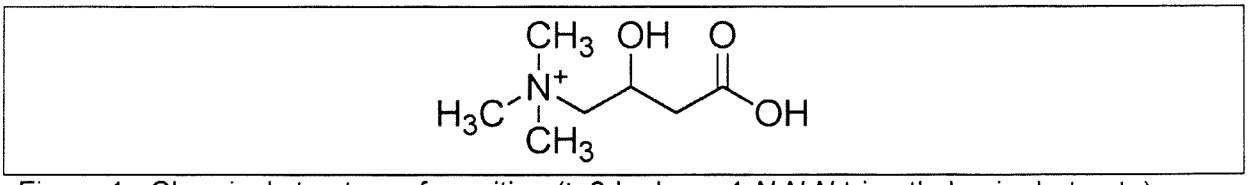

Figure 1. Chemical structure of carnitine (L-3-hydroxy-4-*N,N,N*-trimethylaminobutyrate)

1.2 Esterification of carboxylic acids to CoA and carnitine

The primary function of carnitine in living organisms is to act as a shuttling molecule in order to transfer activated acyl groups across intracellular membranes. The activation of carboxylic acids through esterification to CoA is a common biochemical mechanism which cells use to make these compounds available for metabolic processes. In eukaryotes, however, there are physiological and metabolic obstacles the cell has to overcome. First, the organellar membranes are impermeable to acyl-CoA and secondly, activation of the acetyl groups removes free CoA from the limited pool available in the different cellular compartments (Bremer, 1983). To overcome these obstacles, the cell trans-esterifies acyl groups to carnitine to form acylcarnitines, which can then be transferred in-between intracellular compartments. This is a reversible reaction and the acylcarnitines can react again with free CoA to form carnitine and acyl-CoA. This process forms a shuttle mechanism and is therefore called the carnitine shuttle. Through the carnitine shuttle, the CoA pools can also be balanced in the different cellular compartments (Bremer, 1983).

RCO-S-CoA + carnitine-OH ⇔ RCO-O-carnitine + CoA-SH

On the molecular level, the carnitine shuttle requires specific carnitine/ acylcarnitine translocases in the organellar membranes that can channel these compounds in and out of the cellular compartments. In addition, enzymes are required in these compartments that can reversibly acylate carnitine. These enzymes are called carnitine acyltransferases and they will be discussed in more detail with regards to their structure and function.

1.3 Carnitine acyltransferases

The yeast *Saccharomyces cerevisiae* only possesses one class of carnitine acyltransferase, namely carnitine acetyltransferase (Bremer et al., 1983; Kispal et al., 1993; Schmalix and Bandlow, 1993; Swiegers et al., 2001). On the other hand, mammalian cells possess several classes of carnitine acyltransferases. The different classes are distinguished by the chain-length of the carboxylic acids that are esterified to carnitine by the enzyme. This can either be short-chain carboxylic acids e.g. acetyl or different lengths of long-chain carboxylic acids e.g. octanoyl and palmitoyl. The carnitine acyltransferases found in mammalian cells are carnitine palmitoyltransferase 1 and 2, carnitine octanoyltransferase and carnitine acetyltransferase (Bremer, 1983). These enzymes can be sub-classified in two groups based on their inhibition and non-inhibition by malonyl-CoA. The malonyl-CoA sensitive acyltransferases have their catalytic sites facing the cytosol, the exclusive location of malonyl-CoA (Frazer et al., 1997; Zammit et al., 1997; Zammit et al., 1998). Interestingly, there is an inverse relationship between fatty acid oxidation (a pathway where carnitine acyltransferases play an important role) and malonyl-CoA concentration (McGarry and Foster, 1979). In mammals, malonyl-CoA serves as an important regulatory target for insulin, which stimulates an increase in the concentration of malonyl-CoA whereas glucagon results in the decrease of malonyl-CoA concentration, making this an important area of diabetes research (Beynen et al., 1979). A schematic representation of the localisation and function of carnitine acyltransferases in mammalian cells is given in Figure 2.

1.3.1 Carnitine acetyltransferases

Carnitine acetyltransferases (CATs) are found in most eukaryotic organisms. CATs catalyse the reversible reaction between acetyl-CoA and carnitine to form acetylcarnitine and free CoA.

acetyl-CoA + carnitine ⇔ acetylcarnitine + CoA

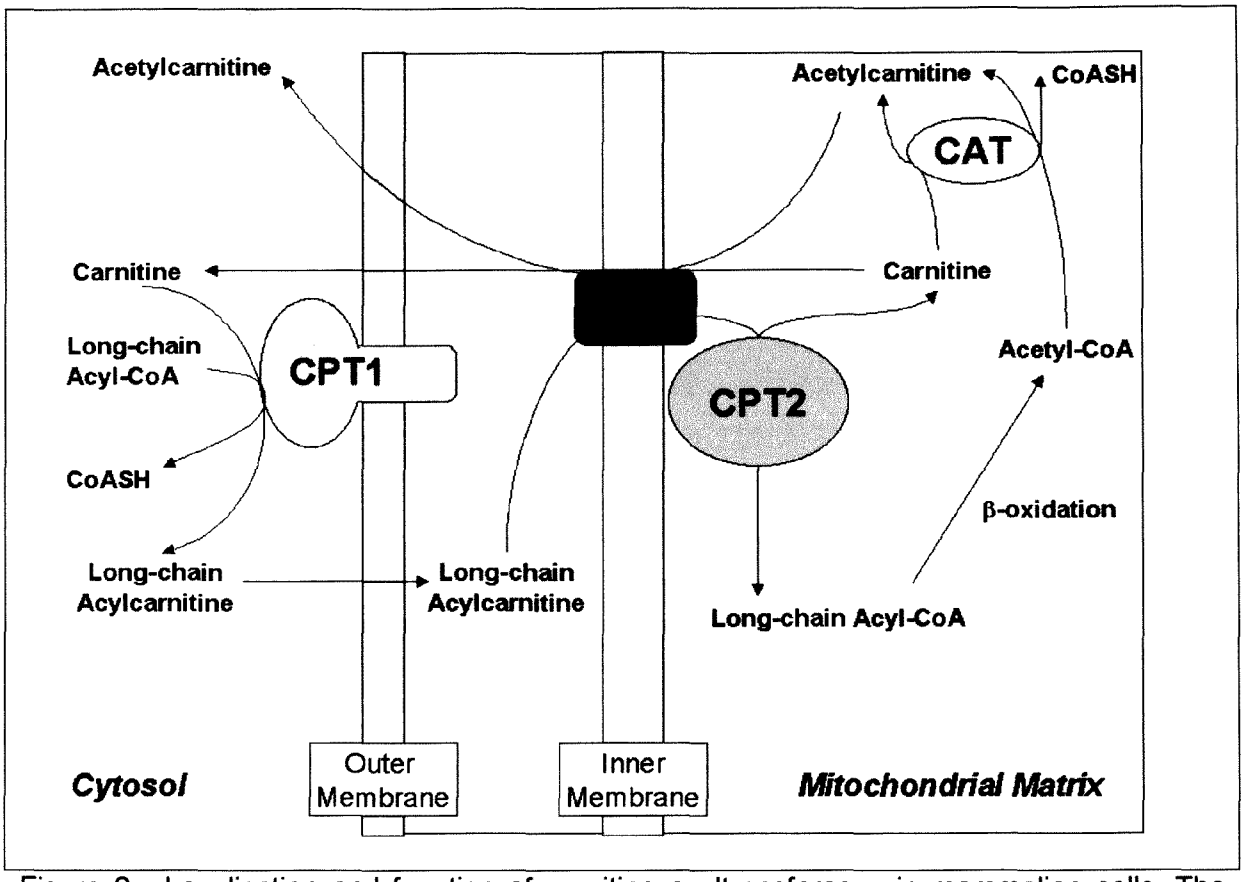

Figure 2. Localisation and function of carnitine acyltransferases in mammalian cells. The carnitine palmitoyltransferase 1 (CPT1) is located in the outer-mitochondrial membrane and catalyses the reaction of long-chain acyl-CoA to long-chain acylcarnitine which is then tranlocated by the mitochondrial inner-membrane CACT into the mitochondrial matrix. The carnitine palmatoyltransferase 2 (CPT2) enzyme is attached to the inner-mitochondrial membrane and converts the long-chain acylcarnitine to long-chain acyl-CoA, releasing carnitine. Long-chain acyl-CoA is oxidised to acetyl-CoA which can be converted by the mitochondrial matrix CAT to acetylcarnitine and translocated out of the mitochondria and/or it can be used in the TCA cycle for energy production.

In some organisms, such as the yeast *S. cerevisiae*, CATs are the only carnitine acyltransferases present (Kispal et al., 1993; Schmalix and Bandlow, 1993; Swiegers et al., 2001). CATs are relatively easily extracted and purified from living material and have been isolated from pigeon breast (the commercially available CAT) and yeast such as *Candida tropicalis* (Chase et al., 1967; Ueda et al., 1982). In rat liver cells, the enzyme is found in the lumina of the mitochondria, peroxisomes and also in mircrosomes, with most activity being associated with the mitochondria (Markwell et al., 1973; Kahonen, 1976). The presence of a cytosolic CAT has not been confirmed in mammalian cells. It has been concluded in a study on heart cells that, if cytosolic CATs are present, their activity is probably very low (Abbas et al., 1998). In other organisms such as the filamentous fungi *Aspergillus nidulans* and the yeast *S. cerevisiae*, it has however been shown that cytosolic CATs are present and active (Stemple et al., 1998; Swiegers et al., 2001; Kroppenstedt et al., unpublished).

In humans, a single gene appears to encode peroxisomal and mitochondrial CAT (Corti et al., 1994). The differential splicing of the mRNA results in two transcripts, with the longer transcript containing the N-terminal mitochondrial targeting signal. After mitochondrial matrix protease modification the mitochondrial CAT is smaller (67,5 kDa) compared to the peroxisomal CAT (69 kDa). Both enzymes contain the peroxisomal C-terminal AKL peroxisomal target, suggesting that the mitochondrial signal overrides the peroxisomal signal (Corti et al., 1994). In the yeast *S. cerevisiae*, a similar transcriptional regulation of the *CAT2* gene, encoding carnitine acetyltransferase, exists (Elgersma et al., 1995). As in humans, the *CAT2* mRNA is differentially spliced in order to translate peroxisomal and mitochondrial targeted CATs. However, evidence for mitochondrial modification of the mitochondrial CAT has not been presented.

In mammalian cells, medium- and long-chain fatty acid β-oxidation (degradation of fatty acids) occurs in the mitochondria. However, very-long-chain fatty acids first undergo partial β-oxidation in the peroxisome and the resulting catabolic product, acetyl-CoA, is transferred from the peroxisome to the mitochondria. This process is facilitated by peroxisomal and mitochondrial CATs. In contrast, the peroxisome is the sole site for β-oxidation in *S. cerevisiae* (Kunau et al., 1988). The activated acetyl residue is transferred out of the peroxisome through the activity of Cat2p and the carnitine shuttle (van Roermund et al., 1995). The CoA pool in the peroxisomes of eukaryotic cells is limited and CATs are therefore essential for β-oxidation to proceed because they free CoA by removing activated acetyl groups from the peroxisome.

In mammalian cells, acetyl-CoA in the mitochondria originates from only two sources: fatty acid β-oxidation and glucose metabolism (glycolysis). In the second case, pyruvate is transferred from the cytosol through the pyruvate dehydrogenase complex (PDH) leading to the formation of acetyl-CoA in the mitochondria. In addition to these two origins, yeast and filamentous fungi can also recruit acetyl-CoA to the mitochondria from the cytosol. In this case, acetyl-CoA is generated through the metabolism of ethanol and acetate. This may explain the presence of a cytosolic CAT in these organisms.

Recent work on CATs in *S. cerevisiae* has indicated some interesting phenotypes of CAT deletion mutants when cells are grown on non-fermentable carbon sources (e.g. fatty acids, ethanol, acetate) (van Roermund et al., 1995; Swiegers et al., 2001). In yeast, unlike in mammals, the carnitine shuttle is not the only pathway through which peroxisomal and cytosolic activated acetyl groups can be transferred to the mitochondria. The glyoxylate cycle, which is not present in mammals, can incorporate two acetyl-CoAs and release succinate, which can be transferred to the mitochondria. Therefore, strains of *S. cerevisiae* that have a disrupted glyoxylate cycle because of the deletion of the citrate synthase, encoded by *CIT2*, are dependent on L-carnitine, and therefore the carnitine shuttle, for growth on non-fermentable carbon sources. Disruption of any of the three identified CATs in the Δ*cit2* mutant results in a severe

growth defect on non-fermentable carbon sources, even in the presence of carnitine (Swiegers et al., 2001). Indeed, *S. cerevisiae* has three CAT encoding genes: (i) *CAT2*, encoding the mitochondrial CAT (Kispal et al., 1993; Elgersma et al., 1995); (ii) *YAT1*, encoding an outer-mitochondrial CAT and (iii) *YAT2*, encoding the cytosolic CAT; (Swiegers et al., 2001; Kroppenstedt et al., unpublished). A presentation of the carnitine acetyltransferases in *S. cerevisiae* is shown in Chapter 3, Figure 4.

The differences between yeast and mammalian metabolism are important factors influencing the carnitine acyltransferase function in these cells. To summarise, the differences are as follows: (i) yeast metabolise acetate and ethanol to acetyl-CoA in the cytosol whereas mammalian cells do not use these compounds as carbon source; (ii) yeast have the unique glyoxylate cycle functioning in the cytosol or peroxisome; and (iii) yeast β-oxidation is restricted to the peroxisome whereas mammalian β-oxidation is located primarily in the mitochondria.

In mammalian cells, β-oxidation of fatty acids in the mitochondria has important regulatory effects on their activation in the cytosol, which could help explain why there are no CATs in this compartment. Studies have shown that during high rates of fatty acid β-oxidation, there is a major increase in acetylcarnitine concentration in the mitochondria (Zammit, 1981). This results in the efflux of a large amount of acetylcarnitine from the mitochondria into the cytosol. Hypothetically, if CATs were present in the cytosol, they would catalyse the formation of large amounts of carnitine and acetyl-CoA. Subsequently, the limited pool of free CoA would be depleted. In contrast to yeast, free CoA is needed in the cytosol of mammalian cells for the activation of fatty acids. After activation, fatty acids can then enter the mitochondria to be catabolised through β-oxidation. The depletion of free CoA would inhibit β-oxidation of fatty acids because there would not be enough CoA left for activation. Moreover, the high concentration of acetyl-CoA in the cytosol would provide building blocks for the formation of the cytosolic malonyl-CoA. Malonyl-CoA inhibits the function of outer-mitochondrial carnitine palmitoyltransferase 1, which is essential for fatty acid oxidation. Therefore, the presence of a cytosolic CAT would be counter productive in conditions where the cell needs to oxidise large amounts of fatty acids. Without the cytosolic CAT, as is the case, acetylcarnitine will either leave the cell or be transported to organelles such as the peroxisome where it can be converted to acetyl-CoA, thereby avoiding the formation of this compound in the cytosol.

1.3.2 Carnitine palmitoyltransferases

In 1955, work on carnitine acyltransferases was initiated through research showing that the oxidation of the long-chain fatty acid, palmitic acid, could be performed by liver homogenates (Fritz, 1955). This finding created interest in the field and later studies identified carnitine as a mediator of fatty acid oxidation. It was shown that

carnitine palmitoyltransferase enzymes were required for the transfer of long-chain acyl-CoA from the cytosol to the mitochondria. In this way, the problem of impermeability of the mitochondrial inner-membranes to CoA esters could be overcome (Fritz, 1963). After this work, the field did not draw much attention until it was discovered in 1973 that a defective carnitine palmitoyltransferase (CPT) could give rise to debilitating human diseases (DiMauro and DiMauro, 1973). This initiated a major thrust of research on CPT enzymes, in particular regarding their structure, function and regulation.

1.3.2.1 Carnitine palmitoyltransferase 1

1.3.2.1.1 Primary structure and topology

The mitochondrial carnitine palmitoyltransferase 1 (CPT1), situated in the outer-mitochondrial membrane, is by far the most studied CPT. It has an interesting structure which is important for function and regulation.

There are two isoforms of CPT1, the liver (L) and the muscle (M) isoforms, each encoded by its own gene. In addition, there has been a recent report indicating that the mRNAs of the L-CPT1 and M-CPT1 undergo differential splicing, subsequently resulting in different sized CPTs translated from each of the two genes (Yu et al., 1998). In rats, the L-CPT1 is found in the liver, kidney, pancreatic cells, ovary, spleen, intestine and brain while the M-CPT1 is found in the heart, skeletal muscles and adipose tissue (Brown et al., 1997).

The amino acid sequences of M-CPT1 and L-CPT1 have a similarity of 63% to each other. There are also regions in both isoforms with high homology to CPT2. The primary structure of M-CPT1 consists of a short N-terminal domain, two transmembrane domains separated by a hydrophilic loop and a catalytic C-terminal. This correlates with the membrane topology determined for L-CPT1 expressed in hepatic cells (Fraser et al., 1997). The CPT1 secondary structure can be divided into four domains. The N-terminal and catalytic C-terminal domains are on the cytosolic side of the membrane. The loop, situated between the two transmembrane regions, faces the mitochondrial inner-membrane space. Both the acyl-CoA and the inhibiting malonyl-CoA sites of the CPT1 enzyme face the cytosol (Fraser et al., 1997). An illustration of the membrane topology and interactions is shown in Figure 3.

Recently, the structure function relationship of CPTs was examined through heterologous expression of CPTs in the yeasts *Pichia pastoris* and *S. cerevisiae*. These organisms are devoid of CPT activity and are able to express mammalian CPTs functionally, making the analysis of enzyme activity simple. The rat L-CPT1 cDNA was successfully expressed in *P. pastoris* and it was confirmed in this system that the enzyme is catalytically active, malonyl-CoA sensitive and reversibly inactivated by detergents such as Triton-X-100 (de Vries et al., 1997). It is important

to consider the last point when enzyme activity assays are conducted on enzymes expressed in yeast as detergents can inhibit their activity. However, in *S. cerevisiae* initial expression studies were not conclusive because not enough active enzyme was expressed to evaluate its properties accurately (Brown et al., 1994). Improved expression of CPT1 in *S. cerevisiae* has recently been reported (Prip-Buus et al., 1998). In this work, comparisons were also made between the CPT1 expressed in *S. cerevisiae* and the CPT1 expressed in *P. pastoris* and rat liver mitochondria (RLM). The kinetic parameters of the enzymes expressed in the three systems indicated similar Km values for carnitine. However, the Km and Vmax values for palmitoyl-CoA were higher for the *P. pastoris* and *S. cerevisiae* expressed enzymes compared to that of the RLM enzyme. Besides the analysis of kinetic parameters, the successful expression of CPT1 enzymes in yeast can also be used as an effective system to study structure/function relationships through targeted mutagenesis and subsequent analysis of function. For instance, the location of the binding sites of CPT1 for its substrates and the inhibiting malonyl-CoA has been determined using *P. pastoris* as an expression system. These sites were mutated and subsequent analysis revealed which substrate or inhibitor was not able to bind (de Vries et al., 1997, Zhu et al., 1997a; Zhu et al., 1997b).

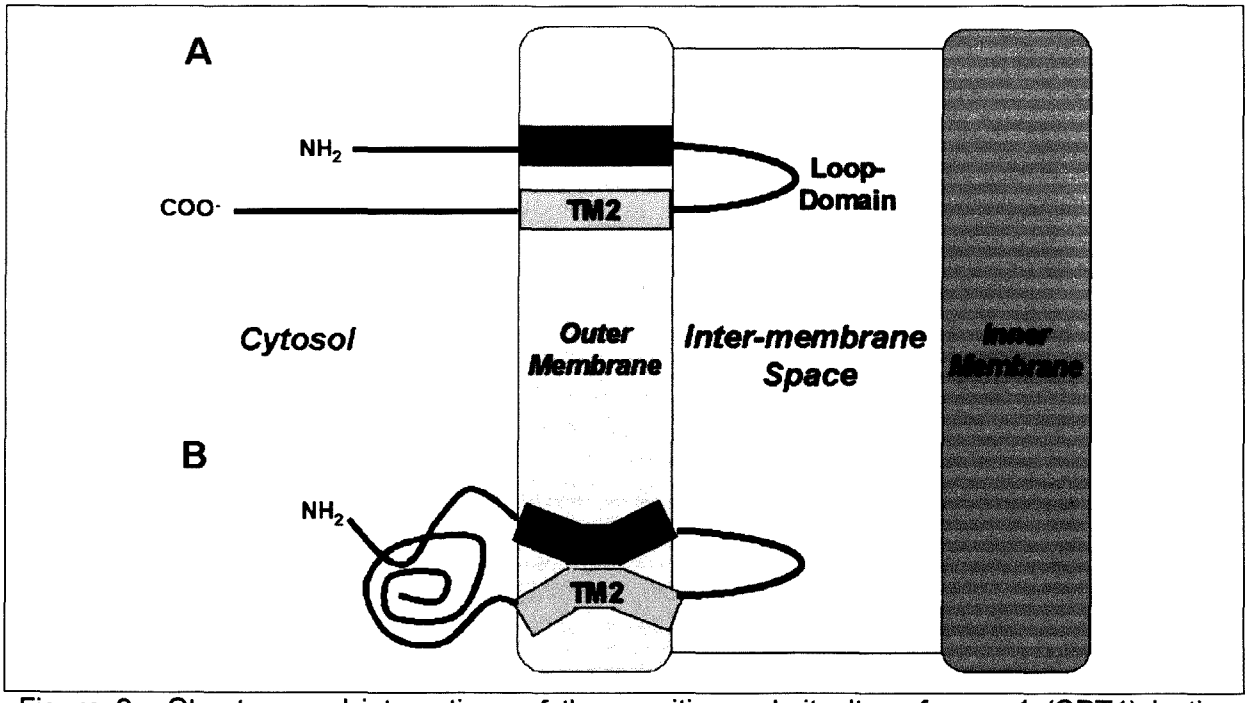

Figure 3. Structure and interactions of the carnitine palmitoyltransferase 1 (CPT1) in the outer-mitochondrial membrane. (A) The enzyme consists of two transmembrane domains (TM1 and TM2) connected by a loop domain in the inter-membrane space. The cytosolic side consist of a short N-terminal domain and a long C-terminal domain. Most of the protein is on the cytosolic side of the membrane and the substrate and malonyl-CoA binding sites are situated in this region. (B) The N-terminal domain interacts with the C-terminal domain to maintain the tertiary structure for optimal catalytic activity and sensitivity to malonyl-CoA. Interactions between the TM1 and TM2 domains can affect catalytic activity (Zammit, 1999).

Although expression of CPTs in heterologous systems is useful, the foreign environment can have some negative influences on the enzyme. In order to mimic conditions in the mitochondrial membrane, CPT1s have recently been reconstituted in liposomes and their activity analysed (McGarry and Brown, 2000). Six CPT1s were histidine-tagged and expressed in *P. pastoris*. The enzymes were isolated and purified using a metal-chelate affinity matrix. The initial isolation procedure from the yeast made use of Triton X-100, which is known to inhibit CPT1 activity because this compound disrupts the outer-mitochondrial membrane. Indeed, enzymatic analysis indicated that CPT1 activity was almost completely abolished. The purified enzyme was then reconstituted in liposomes and showed a 200 to 400-fold increase in enzymatic activity. Comparison of the enzymatic activity of CPT1 in liposomes with that of CPT1 enzyme in rat liver and *P. pastoris* mitochondria, showed that the enzyme behaved much more like the native rat liver mitochondrial enzyme that the heterologous *P. pastoris* enzyme. These data support the use of this system to study CPT1 function (McGarry and Brown, 2000).

The study of the structure/function relationship of CPT1 led to the determination of the specific topology of the enzyme in the outer-mitochondrial membrane and the factors influencing its function. Two important discoveries were that the substrate and malonyl-CoA binding sites, as well as the N- and C-terminal domains, face the cytosol. In addition, it was found that interactions between the N- and C-terminal domains are important in maintaining the tertiary structure of CPT, which then ensures optimal activity of the catalytic domains (Zammit et al., 1997). Removal of only nine N-terminal amino acids resulted in a 60% loss of activity and made the enzyme malonyl-CoA insensitive (Fraser et al., 1997). Changing the N-terminal amino acids of CPT1 can also influence the kinetics of the enzyme. This was demonstrated by replacing the distinctive N-terminal segment of ovine M-CPT1 with that of rat M-CPT1 (Price et al., 2003). As a result, the Km for the *P. pastoris* expressed enzyme was altered for palmitoyl-CoA (decreased) and carnitine (increased). Other workers focussed on the C-terminal region of M-CPT1 by making a series of deletions and substitutions in this region and measuring the activity of the *P. pastoris* expressed enzymes (Dai et al., 2003). It was shown that leucine-764 is essential for catalysis. Substitution of leucine with alanine resulted in a 40% loss of activity while substitution with arginine resulted in 84% loss of activity. Interestingly, substitution with a valine had no effect on activity. Secondary structure analysis showed that amino acid residues 744-764 form a coiled-coil alpha helix in the extreme C-terminal region and that this may be important for folding and subsequent activity.

Topological effects of the mitochondrial outer- and inner-membrane itself have an influence on the optimal functioning of CPT1. Previously, it has been indicated that the inner- and outer-mitochondrial membranes have contact sites between them in some places, which would then imply possible contact between CPT1 and the inner-

mitochondrial carnitine palmitoyltransferase, CPT2 (Fraser and Zammit, 1998). Therefore, it has been proposed that channelling of acetylcarnitine into the mitochondria takes place through the CPT1/CPT2 contact sites (Zammit, 1999). In support of this hypothesis, these contact sites represent 5-10% of the surface area of the outer-mitochondrial membrane but contribute about 40% of both CPT1 and CPT2 activity. Interestingly, the carnitine transporter is not concentrated at the contact sites but is more randomly distributed around the inner-mitochondrial membrane, indicating that it might have a more general role in the reverse uptake and outflow of carnitine and acetylcarnitine (Zammit, 1999)

1.3.2.1.2 Metabolic regulation

In mammals, during starvation and uncontrolled diabetes, the liver increases the production of ketone bodies. This increase is initiated by a fall in the level of insulin (due to low blood glucose levels), resulting in a change in the metabolism of liver cells. In the liver cells, fatty acids can either be synthesised through lipogenesis in the cytoplasma or oxidised through β-oxidation in the mitochondria. A drop in insulin shifts the metabolic balance to the desired oxidation of fatty acid reserves, resulting in the formation of ketone bodies.

A molecule at the centre of this switch in liver cell metabolism is malonyl-CoA, which is exclusively located in the cytosol. Insulin activates the acetyl-CoA carboxylase, the enzyme that catalyses the synthesis of malonyl-CoA, thereby increasing the level of malonyl-CoA in the cytosol. On the one hand, malonyl-CoA is the precursor for fatty acid synthesis, while on the other hand, it inhibits L-CPT1, which initiates fatty acid oxidation in the mitochondria (McGarry and Foster, 1980; McGarry et al., 1989). During high insulin conditions (high blood glucose), the concentration of malonyl-CoA increases in the liver cells, thereby inhibiting CPT1. This increase in malonyl-CoA concentration also makes it availability for lipogenesis, which results in an increase of fatty acids synthesis for storage as energy reserves. During low insulin conditions (low blood glucose), the concentration of malonyl-CoA drops, resulting in less substrate available for lipogenesis and less inhibition of CPT1. This shifts the metabolic balance to the oxidation of fatty acid reserves. Interestingly, in these conditions the level of carnitine in the liver also increases, thereby facilitating the oxidation of fatty acids (McGarry and Foster, 1980; McGarry et al., 1989).

The presence of two distinct CPT1 isoforms in mammalian cells is due to the different metabolic needs of specific cells. In comparison to liver cells, heart and muscle cells have a greater need for the oxidation of fatty acids to produce energy. Therefore, the muscle M-CPT1 isoform is regulated differently from the liver L-CPT1. M-CPT1 is much more sensitive to malonyl-CoA and has a much higher affinity for carnitine (McGarry et al., 1983; Saggerson and Carpenter, 1981). In muscle cells, a raise in insulin (as a result of high blood glucose) causes the predictable increase in

malonyl-CoA concentration. Due to the sensitivity of M-CPT1 to malonyl-CoA, the enzyme is immediately inhibited. Metabolism can then quickly be shifted to glucose oxidation in the mitochondria via the pyruvate dehydrogenase complex (Saddik et al., 1993; Stanley et al., 1996). Therefore, the shift to glucose oxidation in muscle cells is much more sensitive and rapid in conditions of high glucose than is the case for liver cells.

The central regulatory role that CPTs play in regulating blood glucose levels through fatty acid oxidation have made them a prime target for developing drugs for type 2 diabetes. Attempts are being made to design selective inhibitors for L-CPT1. These inhibitors would inhibit the function of L-CPT1 and thereby decrease fatty acid oxidation. This would shift metabolism towards glucose oxidation and result in a decrease in blood glucose levels (Anderson, 1998). However, a major problem with these CPT1 inhibitors would be their effect on M-CPT1 in the heart cells. In these cells there is a continuous need for fatty acid oxidation to produce energy, and therefore inhibition of CPT1 would have severe side effects. A possible solution to this problem might be to develop CPT1 isozyme specific inhibitors but this appears a difficult task due to the high protein homology of these enzymes.

1.3.2.2 Carnitine palmitoyltransferase 2

The carnitine palmitoyltranferase 2 (CPT2) is situated on the inner-mitochondrial membrane facing the mitochondrial matrix (Fraser and Zammit, 1998). The catalytic C-terminal section of CPT2 has significant homology to the catalytic C-terminal section of CPT1 but it does not posses the N-terminal and transmembrane domains. CPT2 is not sensitive to malonyl-CoA inhibition, which may be due to its location in the mitochondria since the mitochondria and the mitochondrial inner-membrane space do not contain any malonyl-CoA (Declercq et al., 1987). CPT2 converts acylcarnitine into acyl-CoA, releasing carnitine, which can be transferred out of the mitochondria or converted to acetylcarnitine via CAT before transfer out of the mitochondria (Ventura et al., 1998).

As in the case of CPT1, CPT2 has also been successfully cloned and functionally expressed in *S. cerevisiae* and *P. pastoris* (Brown et al., 1994; de Vries et al., 1997). In these studies, the site directed mutagenesis of the CPT2 gene indicated that changes of the histidine residue 372, the aspartate residue 376 and the aspartate residue 464 to alanine. Expressing these mutated enzymes in *S. cerevisiae* resulted in complete loss of CPT2 activity.

2. Carnitine biosynthesis

One of the first organisms that were shown to biosynthesise carnitine is the filamentous fungi *Neurospora crassa* (Fraenkel, 1953). Elucidation of the biochemical pathway for carnitine biosynthesis started in 1961 when Bremer showed that the methyl groups attached to the amino group of carnitine originate from methionine and not from choline. The following year, it was also shown that γ-butyrobetaine and not γ-aminobutyric acid or γ-dimethylaminobutyrate is the final intermediate before carnitine is formed (Bremer, 1962). The origin of the carnitine backbone stayed an enigma for about ten years before investigators showed that labelled lysine is converted to carnitine in *N. crassa* (Horne et al., 1971; Horne and Broquist, 1973). Since then the complete pathway for carnitine biosynthesis has been established, but surprisingly, the first gene sequence for a carnitine biosynthesis enzyme was only published in 1998 (Vaz et al., 1998). Today, three of the four genes coding for carnitine biosynthesis enzymes are known for various organisms (Vaz et al., 1998; Galland et al., 1999; Vaz et al., 2000; Vaz et al., 2001; Swiegers et al., 2002; Swiegers et al., unpublished; Chapter 5).

This review focusses on the discovery, the enzymatic and genetic characterisation, and the methods of analysis of the carnitine biosynthesis enzymes. One of the long term goals of our group is to develop a *de novo* carnitine biosynthesising strain of *S. cerevisiae*, so particular attention will be given to detailed issues regarding extraction protocols, enzymatic analysis and genetic characterization, in order to provide a complete reference for future work. The biochemical pathway and intermediates of carnitine biosynthesis are shown in Figure 4.

2.1 Enzymes involved in carnitine biosynthesis

In the first step of carnitine biosynthesis, trimethyllysine (TML) is hydroxylated by the trimethyllysine hydroxylase (TMLH; EC 1.14.11.8) to form 3-hydroxytrimethyllysine (HTML). The aldolytic cleavage of HTML forms 4-trimethylaminobutyraldehyde (TMABA) and glycine, in a reaction catalysed by hydroxytrimethyllysine aldolase (HTMLA; EC 4.1.2.'X'). The dehydrogenation of 4-trimethylaminobutyraldehyde by trimethyl-aminobutyraldehyde dehydrogenase (TMABA-DH; EC 1.2.1.47) results in the formation of 4-*N*-trimethylaminobutyrate (γ-butyrobetaine). The last step involves the hydroxylation, at the 3-position, by γ-butyrobetaine hydroxylase (BBH; EC 1.14.11.1). The following sections will describe each of the four enzymes in more detail. A schematic representation of the enzymes involved in carnitine biosynthesis is shown in Figure 5.

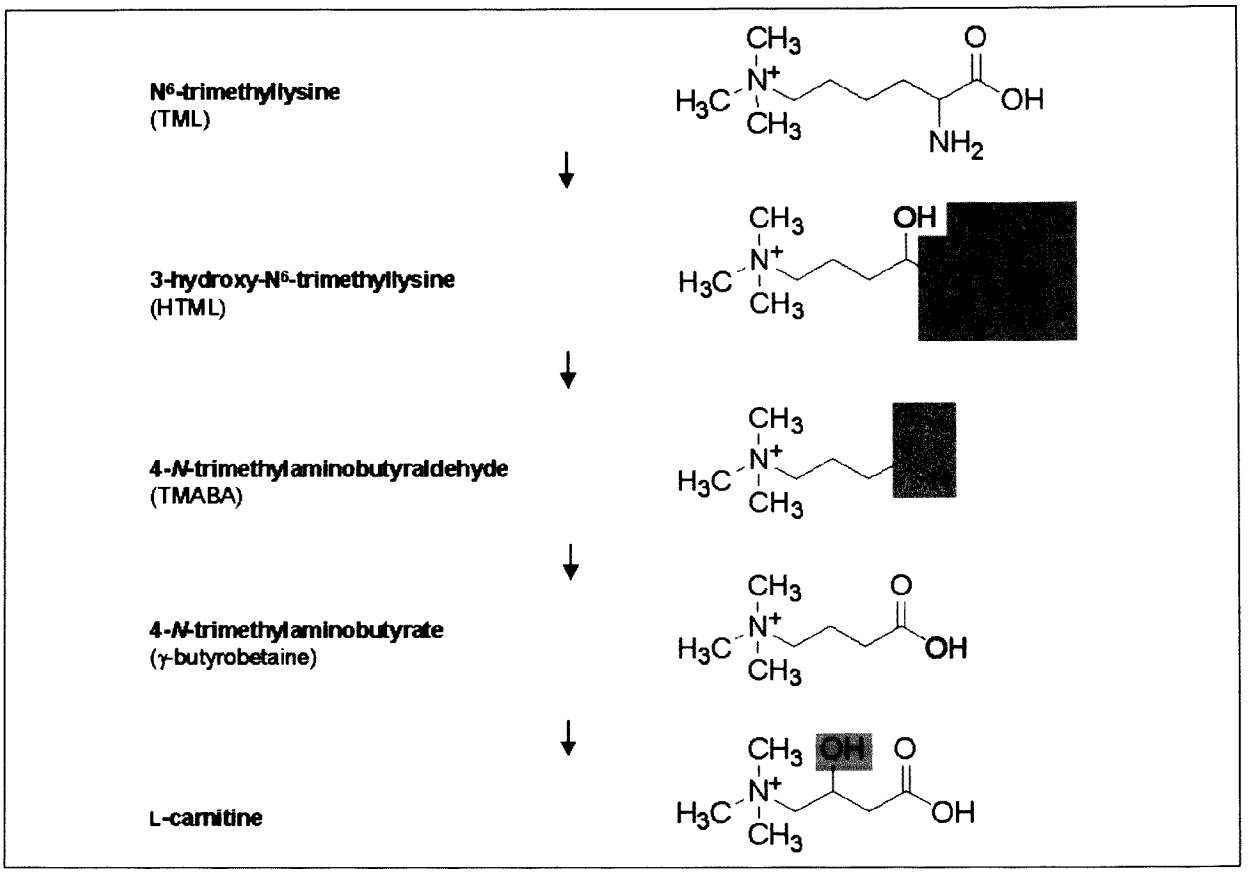

Figure 4. The biochemical intermediates of carnitine biosynthesis.

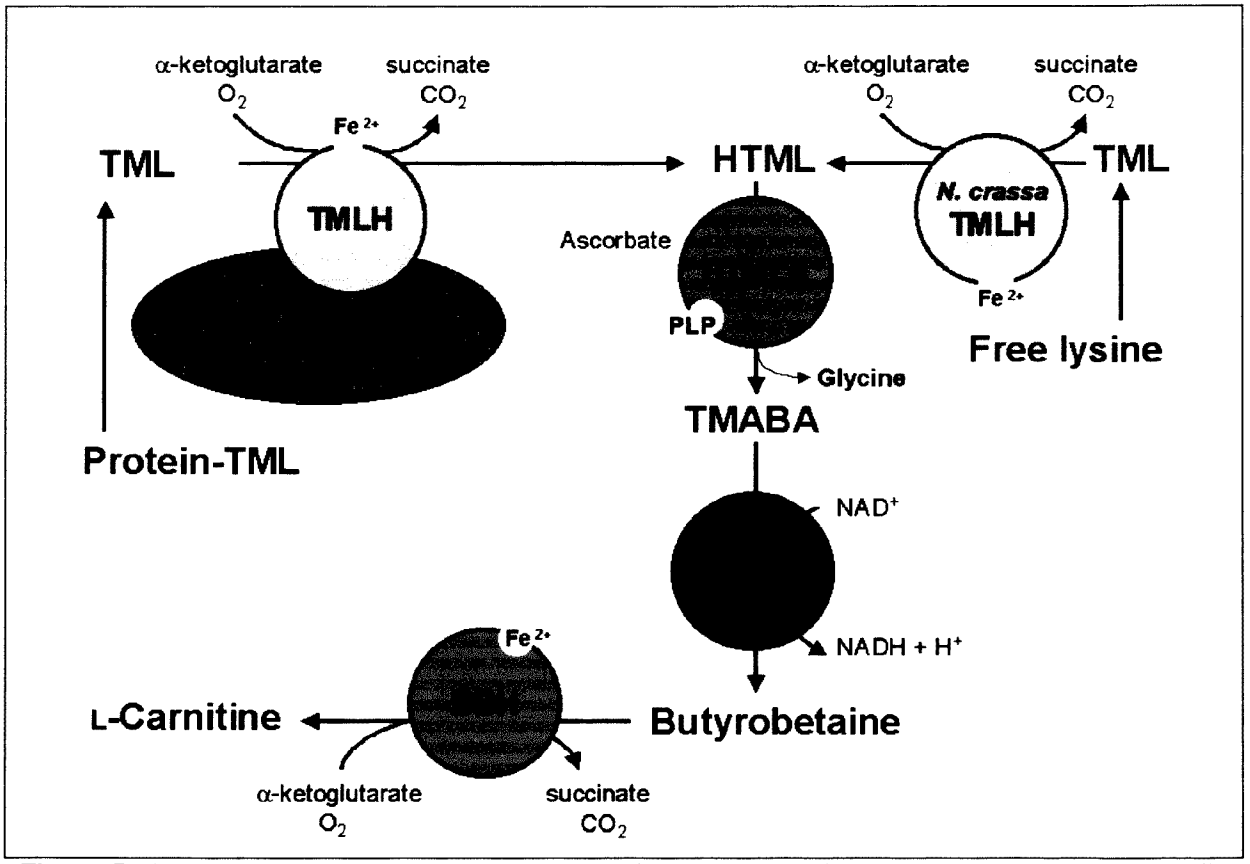

Figure 5. Localisation and function of the carnitine biosynthesis enzymes in mammalian cells and *Neurospora crassa*.

2.1.1 Trimethyllysine hydroxylase (TMLH)

The precursor for the first step in carnitine biosynthesis is the molecule trimethyllysine (TML). The role of lysine in carnitine biosynthesis was first established in 1971, when it was shown that in a *N. crassa* lysine auxotroph, radiolabelled lysine was incorporated into carnitine when the fungi was grown on a carnitine-free synthetic medium (Horne et al., 1971). In animals, certain lysine residues are post-translationally trimethylated in proteins such as myosin, actin and histones by protein lysine methyltransferase (EC 2.1.1.43), and after their degradation, released and used for carnitine biosynthesis (Labadie et al., 1976; Dunn and Englard, 1981). The three methyl groups of TML originate from *S*-adenosylmethionine (Tanphaichitr et al., 1971).

In contrast to animals, free lysine in *N. crassa* is sequentially methylated to form trimethyllysine through the action of a *S*-adenosylmethionine-6-*N*-L-lysine methyltransferase (EC 2.1.1-) (Borum and Broquist, 1977). The enzyme is a soluble monopeptide of 22 kDa and transfers all three methyl groups in a stepwise fashion, with the second transfer faster than the first and the last transfer the faster than the second. In this way the formation of partially methylated lysine is prevented. This is in contrast to animals, where mono- and dimethyllysine can be found in different proteins, but only trimethyllysine is converted to carnitine (Paik and Kim, 1975; Labadie et al., 1976). Unfortunately, no genetic information is available for *N. crassa* *S*-adenosylmethionine-6-*N*-L-lysine methyltransferase, since this gene would be a prime target for manipulation in order to increase the amount of TML produced.

The first step in the identification of TMLH was made in 1976, when it was shown that trimethyllysine injected into a rat was secreted in the urine as hydroxytrimethyllysine (Hoppel et al., 1976). The first report on the hydroxylation of TML was published in 1978 when it was shown that rat liver mitochondria are capable of hydroxylating TML to produce 3-hydroxytrimethyllysine (HTML) (Hulse et al., 1978). Later, Sachan and Hoppel showed that rat kidney homogenates were also able to hydroxylate TML to HTML and at the same time, Sachan and Broquist showed that non-mitochondrial cellular fractions of *N. crassa* were also able to perform this reaction (Sachan and Broquist, 1980; Sachan and Hoppel, 1980).

These workers confirmed through their enzymatic assays that TMLH is a non-haem ferrous-iron hydroxylase, which needs α-ketoglutarate, Fe^{2+} and O_2 as co-factors (Hulse et al., 1978; Sachan and Broquist, 1980; Sachan and Hoppel, 1980). In general, non-haem ferrous-iron hydroxylases require the decarboxylation of α-ketoglutarate to form succinate and CO_2. At the active site of the enzyme, the O_2 reacts to form an oxo-ferryl intermediate (Fe^{4+}=O) which is used to hydroxylate the substrate after which the O_2 is incorporated into α-ketoglutarate resulting in the formation of succinate and CO_2 (Prescott and Lloyd, 2000).

In earlier studies, enzymatic assays were based on the conversion of radiolabelled TML to TMLH in the presence of the essential co-factors α-ketoglutarate, Fe^{2+} and O_2. The radiolabelled TMLH was separated through ion-exchange and radioactivity of the fraction counted with a scintillation counter (Hulse et al., 1978; Sachan and Hoppel, 1980). In these assays, the importance of reducing agents such as ascorbic acid and dithiotreitol was shown, probably to keep the iron in the ferrous state. Sachan and Hoppel showed other miscellaneous factors such as an increase of 20% activity in the presence of catalase and, interestingly, a 2-fold increase in specific activity in the presence of Ca^{2+} when homogenates of kidneys were used (Sachan and Hoppel, 1980). The role of Ca^{2+} in stimulating hydroxylation is not clear but it could assist the transport of TML or α-ketoglutarate across the mitochondrial membrane to the site of TMLH. The Ca^{2+} mediated transport of α-ketoglutarate in rat and rabbit kidney has been reported (Mason et al., 1977). A non-radiolabelled TMLH assay was later developed using ion-exchange and HPLC (Davis et al., 1984). Recently, an assay using HPLC-electrospray tandem mass spectrometry (ESMS) was developed for the determination of TML and other carnitine biosynthesis metabolites in urine (Vaz et al., 2002). The urine is first derivatised to remove interfering compounds and the carnitine biosynthesis metabolites are then separated by ion-pair, reversed-phase HPLC and detected by ESMS. Detection limits were 0.05-0.1 μmol/L.

Before the work by Vaz et al., (2001), there was no sequence information available for TMLH. The enzyme had previously been purified to homogeneity from kidneys but was shown to be very unstable in the particular conditions used, being rapidly inactivated (Henderson et al., 1980). In humans, TMLH activity was shown to be present in liver, muscle, heart and brain but the highest activity was present in the kidney (Rebouche and Engel, 1980). Using a different method, TMLH was purified from rat kidney using liquid chromatography and the enzymatic activity was preserved by the addition of the reducing agents ascorbate and dithiotreitol, as well as by addition of glycerol during purification and storage at -80°C (Vaz et al., 2001). Using gelfiltration and blue native PAGE analysis, it was also shown that TMLH is an 87 kDa enzyme. However, a 43 kDa protein was present in the final purification step and determination of the protein sequence by quadruple time-of-flight MS, indicated that TMLH acts as a homodimer. The short peptide sequences generated by MALDI-TOF MS allowed the identification of the corresponding gene from Expressed Sequence Tag databases and a rat cDNA of 1218 bp, coding for a protein with a calculated mass of 47,5 kDa, was cloned (Vaz et al., 2001). Heterologous expression of the rat cDNA in COS cells and subsequent enzymatic analysis through HPLC-ESMS, confirmed that the gene encodes a TMLH. Interestingly, expression of the gene in *S. cerevisiae* did not result in any TMLH activity. This could be due to the mitochondrial localisation of TMLH. To confirm the localisation of TMLH in the mitochondria, subcellular centrifugation was performed and the TMLH activity fraction

and immuno-reactive material showed that rat TMLH is exclusively located in the mitochondria. Through studying the inactive expressed TMLH in *S. cerevisiae*, it was shown that only a 43 kDa mature protein was present, indicating post-translational modification, probably during import into mitochondria where the mitochondrial import machinery removes the N-terminal presequence. The improper function of this process is possibly the reason for the inactive state of the TMLH expressed in *S. cerevisiae*. Recently, a TMLH gene was cloned from *N. crassa* and the 1413 bp cDNA was successfully expressed in *S. cerevisiae*, showing high TMLH activity (Swiegers et al., 2002). Contrary to mammalian TMLH, the *N. crassa* TMLH is cytosolic, probably explaining the high activity of the heterologous protein (Sachan and Broquist, 1980).

The submitochondrial localisation of mammalian TMLH will have serious consequences for the substrate flow and regulation of carnitine biosynthesis. If mammalian TMLH is located inside the mitochondria, transport systems would be needed to shuttle TML into the mitochondria and hydroxy-TML product out in order for the biosynthesis pathway to continue in the cytosol, where the rest of the enzymes are located. However, an important aspect is that TML in mammals is derived from the hydrolysis of TML-containing proteins, including the mitochondrial cytochrome c. It is the hypothesis of this reviewer that cytochrome c could be hydrolysed in the mitochondria and free TML used as substrate for TMLH. In contrast, *N. crassa* is able to trimethylate free lysine using a cytosolic enzyme and therefore the cytosolic location of its TMLH.

The TMLH proteins cloned from human, mouse, rat and *N. crassa* have very high homology to each other and to butyrobetaine hydroxylases, BBH, the last enzyme in carnitine biosynthesis. The homology of these two enzymes will be discussed in section 2.1.4.

2.1.2 Hydroxytrimethyllysine aldolase (HTMLA)

The second enzyme in the carnitine biosynthesis pathway, hydroxytrimethyllysine aldolase (HTMLA), converts hydroxy-TML to trimethylaminobutyraldehyde and remains enigmatic. No protein sequence or genetic information is known for this enzyme. Rebouche and Engel (1980) generated the only concrete data available for this enzyme by showing that in human tissue extracts, HTMLA activity was almost exclusively contained in the cytosolic fractions. The highest HTMLA activity was found in liver. To date, the enzyme could not be extracted to purity.

It could be that HTMLA is similar to the aldolase serine hydroxy-methyltransferase (SHMT; EC 2.1.2.1). SHMT is a pyridoxal-phosphate dependent enzyme that catalyses the reversible interconversion of serine and tetrahydrofolate to glycine and methylene tetrahydrofolate, generating one-carbon units for methionine,

thymidylate and purine synthesis in the cytoplasma (Girgis et al., 1998). Two isoforms for SHMT exist, of which one is located in the cytosol, while the other is located in the mitochondria. Both these isoforms have been purified to homogeneity from rat liver and have been shown to catalyse the aldolytic cleavage of some β-hydroxyamino acids (Ogawa and Fujioka, 1981). The human cDNA for SHMT have been isolated and cloned through functional complementation of an *E. coli* glyA mutant using a human cDNA library. The cDNA for the mitochondrial and cytosolic enzymes encode proteins of 474 aa, and 483 aa, respectively (Garrow et al., 1993).

It has been indicated that a pyridoxal-phosphate (a derivative of vitamin B_6) enzyme is involved in carnitine biosynthesis, supporting the possible similarity of HTMLA to SHMT. It was shown that in the presence of a vitamin B_6 antagonist, carnitine biosynthesis decreases (Dunn et al., 1982). Like most aldolases, SHMT also uses pyridoxal-phosphate as co-factor. However, the relation of HTMLA to SHMT has not been established to date. It could also be that another specific aldolase might be the HTMLA.

2.1.3 Trimethylaminobutyraldehyde dehydrogenase (TMABA-DH)

Kaufman and Broquist (1977) were the first to suggest that γ-trimethylamino-butyraldehyde (TMABA) is converted by an aldehyde dehydrogenase to form butyrobetaine. Using isotope-labelling experiments in *N. crassa*, they showed that TMABA is an intermediate of carnitine biosynthesis. Later, perfusion experiments showed TMABA being absorbed by rat liver and converted to carnitine via γ-butyrobetaine (Zaspel et al., 1980). At the same time, it was shown that TMABA-dehydrogenase activity was present in the cytosolic fraction of homogenates prepared from liver, kidney, brain, heart, and muscle (Rebouche and Engel, 1980). Concurrently, the TMABA-DH was purified from cytosolic fractions of bovine liver, and it was shown to have maximum activity with TMABA, converting it to γ-butyrobetaine (Hulse and Henderson, 1980). The later workers demonstrated the activity of the pure TMABA-DH by spectrophotometrical measurement of NADH formation. The reaction was started by addition of TMABA to the buffered reaction mixture containing NAD^+ and an increase in absorbance was measured at 340 nm (Hulse and Henderson, 1980).

Recently, the human TMABA-DH was identified and shown to be identical to the human aldehyde dehydrogenase 9 (ALDH9)(EC 1.2.1.19) (Vaz et al., 2000). The human ALDH9 is a cytosolic NAD^+-dependent dehydrogenase belonging to the human aldehyde dehydrogenase gene family (Yoshida et al., 1998). ALDH9 is involved in the synthesis of the well-known neurotransmitter γ-aminobutyric acid (GABA) (Lin et al., 1996; Kikonyogo and Pietruszko, 1996). GABA is synthesised through oxidation of γ-aminobutyraldehyde (the same structure as TMABA without

the trimethyl groups) by ALDH9. Betaine is also synthesised through ALDH9 from betaine aldehyde. This compound can serve as a methyl donor in the synthesis of methionine and regulates osmolarity in the kidney (Petronini et al., 1992; Barak et al., 1996).

The human TMABA-DH (ALDH9) was identified by purification of the TMABA-DH enzyme, which was then subjecting to peptide sequencing (Vaz et al., 2000). The sequences were screened on the Genome Databases and the *ALDH9* gene identified. The cDNAs of human *ALD9* was retrieved from an EST database and cloned into an *E. coli* maltose binding protein (MBP) fusion expression vector. The clones were transformed into *E. coli* and the MBP-ALDH9 purified through an affinity column. Purified rat liver TMABA-DH and the MBP-TMABA was subjected to kinetic analysis. The Km and Vmax values for both enzymes were the same. The affinity for TMABA was shown to be the highest showing a Vmax/Km of 20.83, compared to the values for aminobutyraldehyde and betaine aldehyde, both at 0.71 (Vaz et al., 2000). Because the TMABA-DH enzyme is not only specific for carnitine biosynthesis, homology searches with the protein produces high similarities with different aldehyde dehydrogenases. Although the ALDH9 heterologous protein was functionally expressed in *E. coli*, it has not been functionally expressed in *S. cerevisiae*.

2.1.4 Butyrobetaine hydroxylase (BBH)

The last step in carnitine biosynthesis involves the hydroxylation of γ-butyrobetaine to L-carnitine by the γ-butyrobetaine hydroxylase (BBH). This enzyme was first isolated in crude extracts of rat liver by Lindstedt (1967). Of all the enzymes in the carnitine biosynthesis pathway, BBH is the most studied. Lindstedt showed in preliminary experiments that the enzyme requires molecular oxygen and ferrous iron and that the reaction is stimulated by ascorbate. In these early experiments, carnitine production was measured using radiolabelled γ-butyrobetaine in the reaction mixture (Lindstedt, 1967). Further experiments by the same group revealed that another co-factor is needed, namely α-ketoglutarate (Lindstedt and Lindstedt, 1970).

The BBH protein was later purified from the bacteria *Pseudomonas* sp. AK 1 by ion-exchange, adsorption and molecular-sieving chromatography. By using isoelectric focussing and equilibrium sedimentation, it was shown that the enzyme was purified to homogeneity. Equilibrium centrifugation was used and the molecular mass was determined to be 95 kDa (Lindstedt et al., 1977). In this work, enzyme activity was determined by measuring the $^{14}CO_2$ formed from radiolabelled α-ketoglutarate in the presence of co-factors and α-ketoglutarate. The enzymatic mechanism catalysed by BBH involves the incorporation of one atom of molecular oxygen into carnitine and the other into succinate (Lindblad et al., 1969).

In 1993, the first molecular work on a carnitine biosynthesis enzyme was done through sequencing the protein sequence of the *Pseudomonas* sp. AK 1 BBH through Edman degradation (Ruetschi et al., 1993). In this work, it was shown that the BBH enzyme functions as a dimer with two polypeptide chains, one of 383 residues and the other of 382 residues with molecular masses of 43 321 Da and 43 207 Da, respectively. These two peptides are identical, except for the presence or absence of an N-asparagine residue and contain cysteine residues and 13 histidine residues, amino acids that have been implicated in iron binding.

The BBH enzyme is solely localised in the cytosol (Lindstedt and Lindstedt, 1970; Lindstedt et al., 1982; Galland et al., 1999; Vaz et al., 1999b). The BBH is expressed in various organs such as liver, kidney, brain and possibly testis. Interestingly, BBH activity is found in kidney extracts of human, cat, cow, rabbit, hamsters and Rhesus monkeys but not in kidney extracts of sheep, dogs, mice, rats and Cebus monkeys (Erfle, 1975; Englard and Carnicero, 1978; Englard, 1979; Lindstedt et al., 1982). It is interesting that BBH activity is found in the human brain but not in the brains of any other mammal (Rebouche and Engel, 1980). The reason for the differential expressions is not clear.

Recently, the human cDNA encoding BBH was identified (Vaz et al., 1998). The gene was identified through purification to homogeneity of the protein from rat liver. The protein was then subjected to N-terminal sequencing using Edman degradation. The amino acid sequence was used to screen the genome database and led to the identification of a human cDNA containing an open reading frame of 1161 bp coding for a protein of 387 amino acids with a predicted molecular mass of 44.7 kDa. The cDNA was cloned into a yeast expression vector and the BBH enzyme heterologously expressed in the yeast *S. cerevisiae*. Enzyme activity was determined by using the homogenate of the transformed yeast and the measurement of carnitine in a radioisotopic assay. High activity was obtained in excess of 1 nmol/min/mg. The enzyme did not contain any peroxisomal or mitochondrial targeting signals, supporting the cytosolic location of BBH. Using the full-length cDNA as probe in Northern blot analysis, the expression of BBH in different human tissues could be studied. Expression of the BBH cDNA was found in kidney, liver and brain but not in heart, skeletal muscle, colon, thymus, spleen, small intestine, placenta, lung and peripheral blood leukocytes. Highest expression was found in kidney and the lowest in the liver. Expression in brain was very low (Vaz et al., 1998).

Galland and co-workers cloned and characterised the rat liver BBH (Galland et al., 1999). The cDNA contained an open reading frame of 1161 bp encoding a protein of 387 amino acids with a molecular weight of 44.5 kDa. The rat BBH is expressed in the liver, and, contrary to humans, also in the testis and the epididimus. The size of the mRNA in the epididimus (4.5 kb) and testis (3.5 kb) is bigger than the mRNA in the liver (1.9 kb), which could be due to alternative splicing. Interestingly, BBH was only expressed in the adult rats after weaning of the baby rats (Galland et

al., 1999). This corresponds to data shown for human liver, showing low BBH activity at birth and high activity in adults. However, high BBH activity is found in kidneys at birth (Olson and Rebouche, 1987).

Recently, the BBH gene of *N. crassa* was cloned and expressed in *S. cerevisiae*. The enzyme was able to endogenously synthesize carnitine in transformed yeast grown on media containing γ-butyrobetaine. Interestingly, the carnitine-dependent strain, Δ*cit2*, was able to grow on non-fermentable carbon sources containing γ-butyrobetaine when the BBH was expressed in yeast, indicating that carnitine biosynthesis takes place. The presence of intracellular carnitine was confirmed using electrospray mass spectrometry (Swiegers et al., unpublished; Chapter 6)

The BBH enzymes have high protein homology to each other and to the first carnitine biosynthesis enzyme, TMLH. This is because both enzymes are non-haem ferrous-iron hydroxylases, requiring the same co-factors, α-ketoglutarate, Fe^{2+} and molecular oxygen. BLASTp searches with BBH and TMLH does not find similarities with other proteins, indicating that these enzymes form a separate family of α-ketoglutarate dependent, non-haem ferrous-iron hydroxylases. Two homologous proteins are found in humans, rats, mouse, *Caenorhabditis elegans*, *Drosophila melanogaster* and *N. crassa*. Interestingly, in *S. cerevisiae*, a single weak homologous protein encoded by *YHL021c* is found. However, this gene does not encode for a TMLH or BBH (Swiegers et al., unpublished; Chapter 5). Alignments of BBH and TMLH proteins are shown in Figure 6.

The filamentous fungi *N. crassa* was important in establishing the biochemical pathway for carnitine biosynthesis. The fungal genome contains two homologues genes, one coding for TMLH and the other coding for BBH (Swiegers et al., 2002; Swiegers et al., unpublished). An important area of research in future would be the studying of organisms with disruptions in carnitine biosynthesis enzymes. In this instance, *N. crassa* would be a good candidate for these studies.

2.2 Regulation of carnitine biosynthesis

In order to genetically engineer microorganisms to over-produce carnitine, a good understanding of the regulation of the biosynthesis pathway would be essential. However, nothing is known about the regulation of carnitine biosynthesis in carnitine producing yeast and filamentous fungi. This section of the review therefore focuses on the regulation of carnitine biosynthesis in human, rat and mouse models.

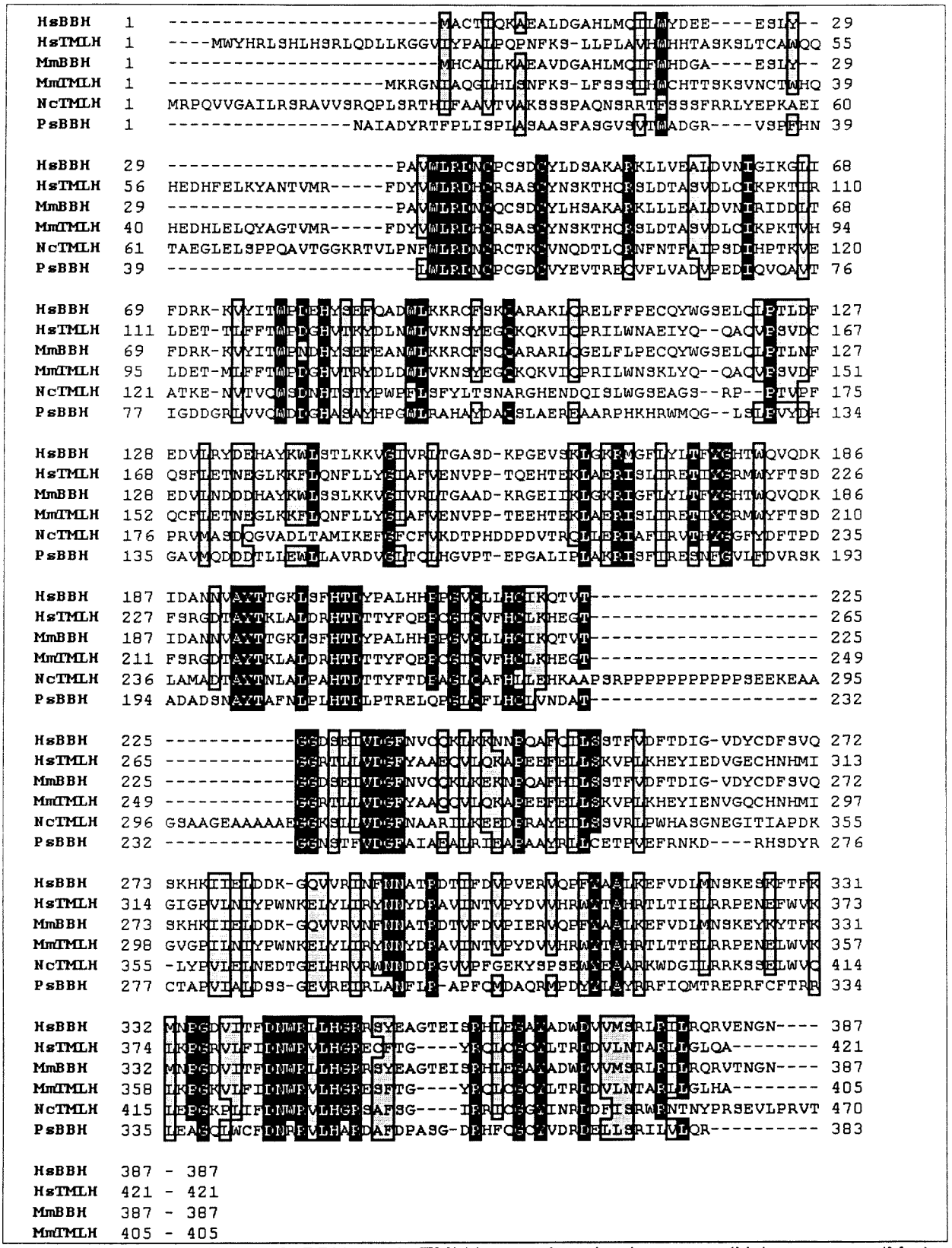

Figure 6. Homology of BBH and TMLH proteins in humans (Hs), mouse (Mm), *Pseudomonas* (Ps) and *Neurospora crassa* (Nc).

2.2.1 Regulation by intermediates

In 1986, Rebouche and workers showed that trimethyllysine (TML) is rate limiting for carnitine biosynthesis in rats. In these experiments, rats that were given TML (but no dietary carnitine) excreted more carnitine in the urine than those that were not fed TML. The rate of carnitine excreted also increased with increasing doses of TML. An increase in carnitine secretion of up to a 100-fold was measured and even at the highest levels of oral TML supplementation, carnitine excretion still increased (Rebouche et al., 1986). Earlier, Rebouche also showed that administration of γ-butyrobetaine to rats could increase levels of carnitine excretion up to 65-fold (Rebouche, 1983). These results suggest that the availability of TML limits the rate of carnitine biosynthesis and that carnitine biosynthesis from TML is not regulated by end-product feedback mechanisms.

In animals, TML is derived from the hydrolysis of proteins containing trimethylated lysine residues (Labadie et al., 1976; Dunn and Englard, 1981). In humans, the liver and muscles together can produce up to 2 μmol of TML in a day through protein hydrolysis, sufficient for carnitine biosynthesis (Rebouche, 1982). In contrast to animals, free lysine in *N. crassa* is sequentially methylated to form TML (Borum and Broquist, 1977). However, there is some evidence suggesting that the carnitine biosythesis enzymes are to some extent being regulated by the intermediates and precursor. Rebouche showed that activity of BBH in rat liver decreases when the diet of rats was supplemented with carnitine. Also, BBH activity increases when rats are fed butyrobetaine (Rebouche, 1982). It is however not clear if this regulation occurs on an enzymatic or gene expression level. The high level of carnitine biosynthesis seen in the experiments where carnitine precursors are added to the diet suggests that the ability of the carnitine biosynthesis enzymes is much greater than required under physiological conditions. Therefore, in mammalian systems, the amount of TML available determines the level of carnitine synthesised.

2.2.2 Pharmacological, hormonal and physiological regulation

Epileptic patients treated with valproic acid tend to suffer from carnitine deficiency. In rats this was confirmed experimentally by showing that the administration of 1.2 mmol/kg valproic acid resulted in decreased levels of carnitine in the liver. However, an increase in the level of butyrobetaine was measured. This would suggest that valproic acid regulates the BBH enzyme directly. However, *in vitro* experiments could not confirm this hypothesis. Physiological experiments showed that valproic acid reduces the amount of the BBH co-factor, α-ketoglutarate, thereby explaining why there is less carnitine synthesised in the presence of valproic acid (Farkas et al., 1996).

The compound 3-(2,2,2-trimethylhydrazinium) propionate (THP or mildronate) inhibits carnitine biosynthesis. It was shown that THP treatment reduced levels of carnitine in the hearts of paced work-performing guinea pigs. Interestingly, THP treatment also protected guinea pigs hearts against hypoxic damage in this study. These findings suggest that THP could be used for the treatment of patients with damaged hearts (Dhar et al., 1996).

The hypolipidemic drug, clofibrate, which stimulates the production of peroxisomes, has been shown to greatly increase liver carnitine and acylcarnitine levels (Paul et al., 1986). In these experiments it was shown that the increase in liver carnitine levels is a direct effect of the increase in carnitine biosynthesis because there was no change in carnitine levels in other tissues and the same amount of carnitine was secreted in the urine. However, clofibrate treatment did increase the level of TML secreted in the urine, suggesting that the increase in carnitine biosynthesis is due to the increased availability of TML.

The hormone thyroxine produced by the thyroid gland has been shown to increase levels of carnitine in the liver (Pande and Parvin, 1980). It was shown that in rats fed 6 mg thyroxine per kg of diet for 10 days, the level of carnitine in the liver doubled, as did the activity of BBH. Recently, it has been shown that the thyroid hormone regulates carnitine levels through modification of the BBH enzyme, resulting in altered Vmax values, and through the regulation of BBH gene expression (Galland et al., 2002).

Physiological conditions such as starvation also have a significant effect on carnitine biosynthesis. In the livers of starved rats, the oxidation of long-chain fatty acids was significantly enhanced and the level of carnitine also increased drastically (McGarry et al., 1975). Starvation results in increased fatty acid oxidation, which is mediated by higher levels of carnitine. It is however not clear if this is due to increased carnitine biosynthesis or due to redistribution of carnitine from other tissues. During starvation, the body also retains carnitine by excreting much less in the urine (Sandor and Hoppel, 1989).

In future, the regulation of carnitine biosynthesis in yeast and fungi such as *N. crassa* would be an interesting field of study. These organisms could act as model systems to study the molecular regulation of carnitine biosynthesis and the possible effects of different drugs and chemicals on the enzymes of carnitine biosynthesis. Researching the possible metabolic reason why some yeast biosynthesise carnitine and others not will also be a fascinating new field.

3. Carnitine in human disease

3.1 Carnitine palmitoyltransferase deficiency

In humans, the carnitine palmitoyltransferases CPT1 and CPT2 are essential for fatty acid oxidation. Therefore, mutations in the genes coding for these enzymes result in severe physiological effects, depending on the site of mutation. Carnitine palmitoyltransferase deficiency is a human disease with debilitating symptoms and inherited defects that are due to mutations in both CPT1 and CPT2 have been described. The outer-mitochondrial CPT1 enzyme deficiency is found in rare cases, while the inner-mitochondrial CPT2 deficiency is more abundant. The reason for the small amount of cases of CPT1 deficiency could be due to the lethality caused by mutations in this enzyme. The first case of CPT1 deficiency was reported relatively recently (Bougneres et al., 1981). Clinical presentation of the illness includes hypoketoic hypoglycemia in infants, which can be fatal if left untreated (Bonnefont et al., 1999). During fasting, the symptoms of patients with L-CPT1 deficiency can be enhanced. In these conditions, glucose needs to be generated in the liver through gluconeogenesis, which depends on fatty acid oxidation through the CPT system. As a result of a deficient CPT1 enzyme, the process becomes inefficient and blood glucose levels drop drastically (hypoglycemia). The reduced capacity of liver fatty acid oxidation also explains the abundant formation of ketone bodies (hypoketoic) (Bougneres et al., 1981). The condition can be treated by administration of triacylglycerols (fatty acids that do not need the CPT system for oxidation) or by maintaining a high carbohydrate and low fat diet. Recently, it has been reported that women carrying a foetus with L-CPT1 deficiency can develop acute liver failure as a consequence (Innes et al., 2000).

In 1998, the basis for L-CPT1 deficiency was first characterised on the molecular level (Ijlst et al., 1998). The human L-CPT1 cDNA has previously been cloned (Britton et al., 1995). The L-CPT1 cDNA was cloned from a patient suffering from L-CPT1 deficiency and expressed in *S. cerevisiae* under the *GAL1* promotor. Enzymatic analysis of the heterologously expressed L-CPT1 revealed that it only had 2% activity when compared to the wild type heterologously expressed L-CPT1 enzyme. Sequencing of the defective L-CPT1 revealed an A to G missense mutation at position 1361 resulting in the change of the aspartate at position 454 to a glycine. In the patient's fibroblasts, an immunoblot revealed a reduced size in the defective L-CPT1, suggesting that the mutation causes the instability of the protein (Ijlst et al., 1998). Later, these findings were confirmed through expression of mutant L-CPT1 in COS cells and subsequent determination of the proteins stability (Brown et al., 2001). In these experiments, mutant L-CPT1 cDNAs of six different patients were expressed in COS cells. The mRNA levels of the six expressed cDNAs were similar to wild type expressed cDNA but immunoblots indicated lower levels of each mutant protein compared to wild type protein.

To date, only 30 families with L-CPT1 deficient enzymes have been described. Interestingly, no patients have so far been described for deficient muscular M-CPT1. This may be linked to the vital role it plays in heart muscle, and a deficient form of M-CPT1 may therefore be lethal in all cases.

In contrast to the limited amount of CPT1 deficiencies, more and more cases of inner-mitochondrial CPT2 deficiency are being reported. It is believed that CPT2 deficiency is the most common cause for abnormal lipid metabolism in humans. The disease was first described in 1973 and since then more than 200 families have been identified with this defect (Di Mauro and Di Mauro, 1973). The disease usually manifests after fasting, heavy exercise or infection resulting in symptoms of muscle pain due to impaired fatty acid oxidation and is often accompanied by renal failure. Additional clinical phenotypes have been described in infants, in particular hypoketoic hypoglycemia, liver failure, heart problems and sudden death (Taroni et al., 1992; North et al., 1995; Bonnefont et al., 1996; Yamamoto et al., 1996).

A number of disease causing mutations in the gene coding for CPT2 have been identified to date. Taroni et al. (1993) identified the first and also most common mutation in CPT2. The mutant CPT2 gene contains a C to T transition at position 439, which changes amino acid 113 from serine to leucine. Expression of the mutant CPT2 in COS cells indicated significant decrease in activity compared to wild type expressed CPT2. The mutant CPT2 was expressed at the same level as wild type but the mutant protein was unstable, resulting in reduced activity (Taroni et al., 1993). Verderio and workers identified a number of additional mutations causing CPT2 deficiency. A proline to histidine substitution was identified in residue 50 of the CPT2 enzyme of five patients. This mutation occurs in the very conserved leucine-proline motive of the family of carnitine acyltransferases. Another rare mutation, resulting in an aspartate to asparagine substitution in position 553, was found in one patient. Both types of mutations were expressed in COS cells and resulted in a significant decrease in CPT2 activity compared to wild type. Biochemical analysis showed that the proline to histidine mutation does not affect the binding of substrate but rather the stability of the protein, resulting in loss of activity (Verderio et al., 1995).

Recently, novel splice site mutations were identified in the genomic gene of CPT2 (Smeets et al., 2003). In this work, a novel splice-site mutation in the splice-acceptor site of intron 2 was found resulting in a 25 bp deletion. In other work, analysis of CPT2 from a patient identified the common serine to leucine substitution at residue 113 on one allele and on the other allele, a novel 4 bp deletion starting at codon 515 thereby creating in a premature stop codon (Deschauer et al., 2002).

The continuous study of the CPT1 and CPT2 mutants could eventually lead to the development of new treatments for genetic diseases of this nature. Especially important is the structure/function relationship of the wild type and mutant CPTs. The possibility of developing drugs that could stabilise the enzymes would be one way of treating this illness. The expression of the protein in heterologous systems with no

CPT activity, such as *S. cerevisiae* and *P. pastoris*, would be of great value and possibly lead to the development of high-throughput screening assays for the identification of new drugs.

3.2 Carnitine translocase deficiency

In humans, carnitine is only biosynthesised in the liver, kidney and to a lesser extent in the brain. It is however essential in organs such as muscles and the heart. Therefore, an adequate system needs to be in place in order to transfer carnitine to these tissues and into the cells (Rebouche, 1992). These tissues take up carnitine from the blood through active transport. Active transport is also involved in the uptake of carnitine from food sources in the intestine and also for renal reabsorbtion in the kidneys (Engel et al., 1981; Rodrigues-Pereira et al., 1988)

Defects in the carnitine transport system result in a severe illness known as systemic carnitine deficiency. In patients with this disease, plasma and carnitine tissue levels are very low due to excessive renal and intestinal wastage coupled with decreased renal reabsorbtion in the kidneys. Patients with this disease develop symptoms of myopathy (muscle wastage) and cardiomyopathy (heart muscle wastage), especially under conditions of starvation when fatty acids become an essential fuel (Tanphaichitr and Leelahagul, 1993). Administration of high doses (in excess of 1 g/day) of carnitine is life-saving for these patients and counters the effect of increased renal secretion of carnitine.

Investigation of the cells of patients with systemic carnitine deficiency indicated a deficient carnitine uptake system (Eriksson et al., 1989; Tein et al., 1990). Tamai and co-workers cloned the first gene coding for a carnitine transporter using a human kidney cDNA library. The gene was named *OCTN2* and encodes a polypeptide of 557 amino acids and is highly expressed in kidney, skeletal muscle, heart, and placenta. The cDNA was expressed in HEK293 cells and resulted in highly efficient sodium dependent carnitine transport. The transporter showed a high affinity for carnitine with a Km of 4.34μM. The OCTN2 transporter is strongly inhibited by acetylcarnitine and butyrobetaine, possibly indicating its role in the transport of these compounds (Tamai et al., 1998).

Recently, mutations in the *OCTN2* gene have been identified from patients suffering from systemic carnitine deficiency (Nezu et al., 1999). Three unrelated patients were investigated. In one patient, a deletion of a 113 bp region containing the start codon of *OCTN2* was identified. In the *OCTN2* gene of the second patient, two separate mutations cause a frame shift and a premature stop codon. In the third patient, the *OCTN2* gene had a splice-site mutation also resulting in a premature stop codon. At the same time it was shown by Vaz and co-workers that mutations in the *OCTN2* gene are indeed responsible for systemic carnitine deficiency. In this work,

fibroblasts of patients were transformed with a wild type *OCTN2* cDNA that restored the ability of these cells to transport carnitine and increased carnitine transport 12-fold. In the three patients tested, two patients had an A to G mutation at position 632 resulting in a tyrosine residue in amino acid position 211 changing to a cystein and one patient had a C to T nonsense mutation in position 844 resulting in a arginine changing to a stop codon in amino acid position 282 (Vaz et al., 1999a). Recent studies have indicated that the OCTN2 transporter is located in the plasma membrane of cells where it can facilitate the import of carnitine into the cells (Tamai et al., 2001).

The human inner-mitochondrial membrane carnitine/acetylcarnitine transporter, CACT, has also been identified (Huizing et al., 1997). The neonatal carnitine/acetylcarnitine deficiency caused by mutations in CACT has severe phenotypes and in most cases result in death. Analysis of the mutant CACT from an affected sibling revealed a 13 bp insertion at position 388 resulting in the skipping of exon 3 and 4 (Yang et al., 2001). In this sibling, CACT enzyme activity was completely absent. Recently, a novel peroxisomal carnitine transporter, OCTN3, was identified and implicated in Crohn's disease (Lamhonwah et al., 2003).

In future, unicellular organisms such as yeast could be used in studying carnitine transporters. The gene coding for the plasma membrane transporter in *S cerevisiae* has been identified as the gene known to code for a general amino acid transporter, *AGP2* (van Roermund et al., 1999). Deletion of this gene in the Δ*cit2* genetic background abolished growth on fatty acids in the presence of carnitine, which confirmed its role in carnitine transport. Intracellular carnitine measurements confirmed that carnitine transport was blocked. In this work and that of other groups, the inner-mitochondrial carnitine/ acetylcarnitine translocase encoded by *CRC1* was also identified, (Palmieri et al., 1999, van Roermund et al., 1999, Swiegers et al., 2001). Deletion of *CRC1* in the Δ*cit2* background displayed the same phenotype as the *AGP2* deletion, showing no growth on oleic acid, even in the presence of carnitine, indicating its vital role in the transport of carnitine and acetylcarnitine across the mitochondrial inner-membrane (van Roermund et al., 1999; Swiegers et al., 2001). The *AGP2* and *CRC1* deletion strains of *S. cerevisiae* could be used for structure/ function analysis of human OCTN2 and CACT.

It is clear that mutations in the genes involved in carnitine transport in humans have severe and debilitating consequences. Interestingly, no case of defects in carnitine biosynthesis genes has ever been identified in humans.

3.3 Carnitine in disease treatment

Besides the use of carnitine to treat systemic carnitine deficiency, it has recently found use in the symptomatic treatment of various unrelated diseases. In the treatment of systemic carnitine deficiency, a large dose of carnitine is needed to counter the effect of excessive renal secretion. However, in other diseases, carnitine is used in different quantities and in some cases derivatised before use

Because of its role in the enhancement of energy production and removal of toxic metabolites during ischemia, it has been suggested that carnitine could be used for the treatment of various cardiac diseases (Retter, 1999). A natural derivative of carnitine, propionyl-L-carnitine, has been shown to improve myocardial (heart muscle) contraction in different experimental models of heart failure. It seems that the beneficial effect of this compound is due to its action on muscle and heart metabolism by increasing the pyruvate flux to the Krebs cycle, especially under conditions of increased workload (Ferrari and Giuli, 1997). Exogenous carnitine treatment has also been shown to be beneficial in other heart conditions such as congestive heart failure, arrhythmia and acute ischemia (Retter, 1999). Some molecular links have been found regarding the prevention of skeletal muscle myopathy by carnitine during heart failure (Vescovo et al., 2002). In this study, treatment of rats with carnitine revealed the inhibition of caspases and tumor necrosis factor-α (TNF-α), both components known to induce apoptosis, suggesting that carnitine inhibits apoptosis. Indeed, previous work has shown that carnitine regulates the activity of caspases and also prevents doxorubicin-induced apoptosis in cardiac myocytes (Andrieu-Abadie et al., 1999; Mutoba et al., 2000).

Carnitine has also recently found use for the symptomatic treatment of immune-mediated diseases such as AIDS and chronic fatigue syndrome. Patients suffering from AIDS often present a degree of carnitine deficiency due to the malabsorbtion of carnitine and the possible negative effect of anti-retroviral drugs on tissue carnitine levels (Famularo and De Simone, 1995). Another immune disease called chronic fatigue syndrome is characterised by persistent debilitating fatigue. Most patients have a deficiency in acetylcarnitine levels in the serum and it has been proposed that there is a relation between acetylcarnitine levels and the onset and severity of this disease (Kuratsune et al., 1994). In both of these diseases, treatment with carnitine was shown to be beneficial to patients.

Acetylcarnitine is also currently under investigation for the treatment of the neuro-degenerative Alzheimer's disease. In these conditions acetylcarnitine is proposed to have a beneficial effect due to its role in brain energy and phospholipid metabolism (Pettegrew et al., 2000). In support of the role of carnitine in the nervous system, it has been shown that acetylcarnitine increases the survival time of rat sensory neurons (Formenti et al., 1992).

The last few years L-carnitine has also been extensively used as a weight loss agent due to its role in fatty acid oxidation. In addition, due to its role in energy metabolism, it is also widely used as an energy booster, mostly in nutritional supplements. Many other nutritional products today contain added carnitine, as do most soy-based commercial infant formulas. However, carnitine is found in high concentrations in foods such as meat and milk, with the highest concentrations found in meat. In fruits and vegetables, carnitine is found in low quantities, making carnitine supplementation beneficial for vegetarians (Carter et al., 1995).

Interestingly, the common bakers yeast, *S. cerevisiae*, does not biosynthesise carnitine (Swiegers et al., 2001). It will be a great nutritional benefit if this yeast could be engineered to produce carnitine in large quantities, adding value to food products such as bread and beverages such as beer and wine.

4. Conclusion

Although the research results that will be discussed in Chapters 3-6 are based on the metabolism of the yeast *S. cerevisiae* and the filamentous fungi *N. crassa*, this literature review indicates how little information on carnitine metabolism and function is available for these organisms. However, the review does state what is currently known about carnitine in these organisms and also points out the application in particular of *S. cerevisiae* in the various experiments designed to elucidate the function of carnitine in mammalian cells, e.g. the expression and analysis of human CPTs in yeast.

It is also of broader interest for the yeast researcher to understand the important differences in carnitine metabolism of mammalian versus yeast cells. This review highlighted the important metabolic role of carnitine in mammalian cells. It is clear that in the yeast system, much can still be learned regarding carnitine acyltransferases, carnitine translocases and carnitine biosynthesis. In *S. cerevisiae*, only five genes have been identified to date for their involvement in the carnitine shuttle and not much is known about their regulation. Of particular interest is the role of the three CATs in the metabolism of non-fermentable carbon sources (Swiegers et al., 2001) (discussed in Chapter 3). In contrast to yeasts, the mammalian system has only one gene coding for a peroxisomal and mitochondrial CAT. This review has offered possible explanations for these differences but this has to be verified experimentally.

Chapters 4 and 5 relates the attempts by our laboratory to try and introduce this pathway in the metabolism of *S. cerevisiae*. This review has looked in depth into what is known regarding carnitine biosynthesis in order to state the obvious challenges in engineering a endogenously carnitine producing yeast but also to serve as a reference for future work in this field. As the reader would have noticed, very little is

known about carnitine biosynthesis, especially on the molecular level. Information on carnitine biosynthesis in unicellular eukaryotes is almost non-existent. The section on the regulation of carnitine biosynthesis in mammalian systems may help to engineer a yeast strain for optimal carnitine production. Of particular interest is the finding that trimethyllysine appears to be the limiting factor in carnitine biosynthesis in mammals.

The importance of carnitine in human health was also highlighted by this review. The severe and debilitating consequences of mutations in genes involved in carnitine dependent metabolic activities is striking. An attempt was also made to indicate the use of yeast such as *S. cerevisiae* to study the mutant CPTs as an easy and effective method of analysis. The possible use of yeast in the high-throughput screening of drugs for treatment of carnitine related illnesses appears promising. The role of carnitine and its derivatives in the treatment of various diseases indicates that there is still much to be learned about the metabolic and physiological function of this molecule. The use of carnitine for disease treatment and nutritional supplementation supports the potential commercial value that a carnitine biosynthesising strain of *S. cerevisiae* would have for the health and well being of human beings.

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CHAPTER 3

RESEARCH RESULTS I

Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: Three carnitine acetyltransferases are essential in a carnitine-dependent strain

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Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: Three carnitine acetyltransferases are essential in a carnitine-dependent strain

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1. Abstract

L-Carnitine is required for the transfer of activated acyl-groups across intracellular membranes in eukaryotic organisms. In *Saccharomyces cerevisiae*, peroxisomal membranes are impermeable to acetyl-CoA, which is produced in the peroxisome when cells are grown on fatty acids as carbon source. In a reversible reaction catalysed by carnitine acetyltransferases (CATs), activated acetyl groups are transferred to carnitine to form acetylcarnitine which can be shuttled across membranes. Here we describe a mutant selection strategy which specifically selects for mutants affected in carnitine-dependent metabolic activities. Complementation of three of these mutants resulted in the cloning of three CAT encoding genes: *CAT2*, coding for an carnitine acetyltransferase associated with the peroxisomes and the mitochondria; *YAT1*, coding for the carnitine acetyltransferase which is presumably associated with the outer mitochondrial membrane and *YER024w* (*YAT2*), which encodes a third, previously unidentified carnitine acetyltransferase. The data also show that (i) L-carnitine and all three CATs are essential for growth on non-fermentable carbon sources in a strain with a disrupted *CIT2* gene, that (ii) Yat2p contributes significantly to total CAT activity when cells are grown on ethanol, and that (iii) the carnitine-dependent transfer of activated acetyl groups plays a more important role in cellular processes than previously realised.

2. Introduction

Carnitine is synthesised by most eukaryotic organisms from lysine as a precursor and methionine or S-adenosyl methionine as a methyl donor (Bieber, 1988). In humans, endogenic synthesis occurs mainly in liver tissues, but is complemented through dietary uptake. Systemic or tissue specific carnitine deficiencies, the latter frequently

due to genetic defects in the plasma membrane carnitine transport system, are debilitating, sometimes life-threatening diseases, highlighting the importance of carnitine in cellular metabolism (Pons and De Vivo, 1995).

Recent data, on the other hand, suggest that the yeast *Saccharomyces cerevisiae* may be unable to synthesise carnitine (van Roermund et al., 1999). Furthermore, wild-type strains appear not to require carnitine under any of the conditions investigated thus far (van Roermund et al., 1995, 1999). However, enzymatic activities that require carnitine, in particular carnitine acetyltransferases (CATs), are present in *S. cerevisiae*, and a plasma membrane transport protein, encoded by *AGP2*, that allows the up-take of carnitine from the extracellular environment has been identified (van Roermund et al., 1999).

The only well-defined molecular role of carnitine in eukaryotic organisms is to facilitate the transfer of activated acyl groups across intracellular membranes. In mammalian cells, this role entails the transfer of long-chain fatty acids to the mitochondria for β-oxidation, as well as the transport of activated medium- and short-chain organic acids from the peroxisomes to the mitochondria (Bieber, 1988). In *S. cerevisiae*, the role of carnitine consists in the transfer of activated acetyl groups of peroxisomal or cytoplasmic origin to the mitochondria (Schmalix and Bandlow, 1993; van Roermund et al., 1995). This process is referred to as the carnitine shuttle, and involves the transfer of the acetyl-moiety of acetyl-CoA, which can not cross organellar membranes, to a molecule of carnitine, the subsequent transport of the acetylcarnitine across membranes and finally the reverse transfer of the acetyl group to a molecule of free CoA for further metabolism.

The transfer of acetyl groups from acetyl-CoA to carnitine and *vice versa* is catalysed by CATs (Bieber, 1988). Two genes coding for these enzymes have been identified in *S. cerevisiae*. The first gene, *CAT2*, codes for a CAT found in the peroxisomes and mitochondria and contributes >99% of the total CAT activity in galactose grown cells (Kispal et al., 1993). The second gene, *YAT1*, codes for a CAT presumably associated with the outer surface of the mitochondria and contributes an estimated 5% to the total CAT activity in acetate and ethanol grown cells (Schmalix and Bandlow, 1993).

When yeast grow on fermentable carbon sources, no clearly identifiable peroxisomal structures can be detected, but when grown on fatty acids such as oleic acid, a proliferation of peroxisomes is observed (Veenhuis et al., 1987). Peroxisomes are required for yeast to catabolise fatty acids, since they are the sole site for β-oxidation, the fatty acid degradation pathway which results in the formation of acetyl-CoA in the peroxisome (Kunau et al., 1988). Van Roermund et al. (1995) showed that *S. cerevisiae* uses two pathways to further utilise this product. The first pathway is the previously described carnitine shuttle, where peroxisomal CAT is thought to catalyse the transfer of the activated acetyl from CoA to carnitine. The acetylcarnitine is transferred to the mitochondria where mitochondrial CAT catalyses

the reverse reaction to form carnitine and acetyl-CoA to enter the tricarboxylic acid (TCA) cycle for energy production. The second pathway utilising peroxisomally produced acetyl-CoA is the glyoxylate cycle, a modified version of the TCA cycle, which, at least in part, takes place in the peroxisome. This cycle results in the net synthesis of the C_4 compound succinate from two molecules of acetyl-CoA, which can then be further utilised for anabolic and catabolic purposes (van Roermund et al., 1995).

The existence of two pathways to further utilise peroxisomally produced acetyl-CoA explains the absence of growth defects on oleic acid media of strains lacking either the *CIT2* gene, which encodes the first enzyme of the glyoxylate cycle, or the *CAT2* gene, encoding the peroxisomal and inner mitochondrial membrane associated CAT. However, a strain disrupted for both *CIT2* and *CAT2* is unable to grow in these conditions (van Roermund et al., 1995), implying that the two pathways can complement each other.

Besides its importance for the acetyl shuttle mechanism between peroxisomes and mitochondria during growth on fatty acids, carnitine also plays a role when yeast cells are grown on other non-fermentable carbon sources like acetate and ethanol. The catabolism of these compounds results in the production of acetyl-CoA in the cytosol which needs to be transferred to the mitochondria for the production of energy. The transfer can take place through the formation of acetylcarnitine in the cytosol and the subsequent transfer thereof to this cellular compartment (Schmalix and Bandlow, 1993; Stemple et al., 1998).

Besides the two CAT-encoding *CAT2* and *YAT1* genes and the carnitine transporter-encoding *AGP2* gene, only one additional gene required for a functional carnitine shuttle, *CRC1*, which encodes a carnitine/acetylcarnitine translocase located in the inner mitochondrial membrane (Palmieri et al., 1999; van Roermund et al., 1999), has thus far been identified. Other genes, which should include translocases located in the peroxisomal and outer mitochondrial membrane, have yet to be identified.

In order to characterise additional carnitine shuttle-specific genes in *S. cerevisiae*, and to analyse the metabolic implications of carnitine-related activities, this paper describes a selection strategy resulting in the isolation of mutants affected specifically in carnitine-dependent metabolic activities. Six mutants, called CPAM for *c*arnitine *p*athway *a*ffected *m*utants, where isolated using this strategy. We furthermore describe the isolation of the genes affected in 5 of the selected mutants through functional complementation. Two of the mutants were complemented by *CRC1*, while three of the mutants were affected in genes encoding carnitine acetyltransferases, corresponding to the previously identified *CAT2* and *YAT1* genes, as well as a newly identified CAT, which is encoded by ORF *YER024w* and was named *YAT2* because of its more significant homology with *YAT1*. While the cloning of *CAT2* and *CRC1* was expected, since the phenotype had been described and the

genes had also been identified in the screen described by van Roermund et al. (1999), no such phenotype has yet been described for *YAT1* and *YAT2*. Our data furthermore show (i) that carnitine is essential for growth on all non-fermentable carbon sources tested in a strain with a disrupted *CIT2* gene; (ii) that *S. cerevisiae* contains three CAT-encoding genes that are essential in these same conditions and are unable to cross-complement each other; (iii) that *YER024w* encodes a previously non-identified CAT which contributes 50% percent to total CAT activity in ethanol-grown cells. Taken together, the data highlight that carnitine plays a more important role in the metabolism of non-fermentable carbon sources than previously realised.

3. Materials and methods

3.1 Yeast strains culture conditions

Yeast strains used in this study derive from FY23 and are isogenic to S288C (Table 1). Yeast were either grown on rich YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] or on minimal YND media, containing 0.67% (w/v) YNB without amino acids (Difco) and 2% (w/v) glucose. Three different media with oleic acid as sole carbon source were used: (i) YNO [0.67% (w/v) YNB without amino acids and 0.1% (v/v) oleic acid]; (ii) YNOC [YNO media supplemented with L-carnitine (10 mg/l)]; and (iii) YNOY [YNO media supplemented with 0.1% (w/v) yeast extract]. Media containing acetate (YNA), ethanol (YNE) and glycerol (YNG) were made with 0.67% (w/v) YNB without amino acids and 3% (v/v) of each carbon source. These media were also supplemented with L-carnitine to a concentration of 10 mg/l (YNAC, YNEC and YNGC). Amino acids (20 mg/l) were added to all media according to the specific requirements of each strain.

3.2 DNA manipulations and construction of plasmids

All plasmids used in this study are listed in Table 1 and primers in Table 2. Standard DNA techniques were applied as described by Sambrook et al. (1989). Sequencing was carried out on an ABI PRISM™ automated sequencer. The *CIT2* gene was amplified by PCR using genomic DNA from strain FY23 as template and primers CIT2F1 and CIT2R1 (Table 2). A 3.4 kb *Hin*dIII-*Eco*RI fragment, containing the *CIT2* gene with its promoter and terminator, was cloned into the *Hin*dIII and *Eco*RI sites of the plasmid YEplac112 (using the *Hin*dIII and *Eco*RI sites introduced in the primers CIT2F1 and CIT2R1). The same fragment was also cloned into the *Hin*dIII and *Eco*RI sites of the plasmid YCplac33. The YEplac112-*CIT2* plasmid was used for the construction of the *CIT2* disruption casette. The major part of the open reading frame (ORF) of the *CIT2* gene was deleted by replacing an internal 1185 bp *Spe*I-*Bal*I

Table 1. Strains and plasmids used in this study

Strains and plasmids	Genotype	Source or reference
Strains:		
FY23	*MATa leu2 trp1 ura3*	Winston et al. 1995
FY23Δ*cit2*	*MATa leu2 ura3 cit2::TRP1*	This study
PSY142	*MATα leu2 lys2 ura3*	
PSY142Δ*cit2*	*MATα leu2 lys2 cit2::URA3*	Kispal et al. 1988
FY23Δ*cat2*	*MATa trp1 ura3 cat2::LEU2*	This study
FY23Δ*cit2*Δ*cat2*	*MATa ura3 Δcit2::TRP1 cat2::LEU2*	This study
FY23Δ*yat1*	*MATa trp1 ura3 Δyat1::LEU2*	This study
FY23Δ*cit2*Δ*yat1*	*MATa ura3 Δcit2::TRP1 yat1::LEU2*	This study
FY23Δ*yat2*	*MATa trp1 ura3 yat2::LEU2*	This study
FY23Δ*cit2*Δ*yat2*	*MATa ura3 Δcit2::TRP1Δyat2::LEU2*	This study
Plasmids:		
YCplac33	*CEN4 URA3*	Gietz and Sugino 1988
YCplac33-*CAT2*	*CEN4 URA3 CAT2*	This study
YCplac33-*CIT2*	*CEN4 URA3 CIT2*	This study
YCplac33-*YAT1*	*CEN4 URA3 YAT1*	This study
YCplac33-*YAT2*	*CEN4 URA3 YAT2*	This study
YDp-L	*LEU2*	Berben et al. 1991
YDp-W	*TRP1*	Berben et al. 1991
YEplac112	2μ *TRP1*	Gietz and Sugino 1988
YEplac112-*CIT2*	*CEN4 TRP1 CIT2*	This study
pBluescript II SK+		Stratagene
PGEM-T-easy		Promega
PGEM-T-easy-*CAT2*		This study
PGEM-T-easy-*CAT2 (P/E)*		This study
pΔ*cat2*	Δ*cat2::LEU2*	This study
pΔ*cit2*	Δ*cit2::TRP1*	This study
pΔ*yat1*	Δ*yat1::LEU2*	This study
pΔ*yat2*	Δ*yat2::LEU2*	This study

fragment with a 0.8 kb fragment containing the *TRP1* gene, isolated from plasmid YDp-W. A 1.6 kb *Apa*I-*Stu*I fragment containing the disruption cassette was isolated and transformed into haploid wild-type FY23 cells. Trp^+ transformants were selected and the disruption of the chromosomal *CIT2* locus confirmed by analysing the PCR product obtained from genomic DNA, using the primers CIT2F1 and CIT2R1. The *CAT2* gene was amplified by the polymerase chain reaction (PCR) method using genomic DNA from strain FY23 as template and primers CAT2F1 and CAT2R1. The PCR product was cloned into pGEM-T Easy plasmid using the Promega pGEM-T Easy Vector System (Promega). A 3890 bp *Pst*I-*Eco*RI fragment containing the *CAT2* gene with its promoter and terminator sequences was cloned into the *Pst*I and *Eco*RI sites of the plasmid YCplac33. This fragment was also cloned into the *Pst*I and *Eco*RI sites of pGEM-T Easy to create plasmid PGEM-*CAT2* (P/E). This plasmid was used for the construction of a disruption cassette, where a 595 bp *Bam*HI-*Bgl*II fragment,

internal to the *CAT2* ORF, was replaced by a 1.6 kb fragment containing the *LEU2* gene isolated from plasmid YDP-L. A 4.1 kb *Dra*I fragment containing the disruption cassette was transformed into FY23 and FY23Δ*cit2* strains. Leu$^+$ transformants were screened and the disruption of the chromosomal *CAT2* locus confirmed by PCR through analysing the PCR product obtained from genomic DNA, using the primers CAT2F1 and CAT2R1.

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
CAT2F1	5'-GACACTGTTCGCCAAATTTCG-3'
CAT2R1	5'-ATAAGCAAGGCACAATATCC-3'
CIT2F1	*5'-AGGTAAGCTTCTCGCTTAGGGTGCGG-3'
CIT2R1	*5'-GAGGAATTCATCAGGTAAAGTTTCCTCGACC-3'
YAT1F1	5'-ATCAGCATCAGCATCAGC-3'
YAT1R1	5'-AGAGGTAATCCAAACGACG-3'
YAT2F1	5'-ATGGATGCTACGGCTTTAGTCG-3'
YAT2R	5'-CAATGGCAAATCTGGTCCGGG-3'

*Underlined sequences indicate introduced restrictions sites

3.3 Random mutagenesis of yeast strain

Transformants FY23Δ*cit2* cells were grown in YPD to an optical density (OD_{600}) of 0.1 corresponding to *ca.* $3x10^6$ cells/ml. Approximatly $4x10^8$ cells were centrifuged and washed twice with sterile water. The cells were suspended in 2 ml 0.1 M sodium phosphate buffer pH 7.0 and treated with 2% (v/v) ethylmethanesulfonate (EMS) for 1 hour at 30°C. The reaction was stopped by adding 1 ml of a 5% (w/v) sodium thiosulphate solution. The survival rates, corresponding to the percentage of cells able to form colonies on rich YPD medium after mutagenesis, were on average 30%. Approximately 30 000 colonies were washed of the YPD agar plates and dilutions were plated on glucose medium (YND). Single colonies (11 000) were picked and streaked in a grid-like fashion on YND agar plates and were allowed to grow for 2-3 days before being replica-plated onto oleic acid media supplemented with L-carnitine (YNOC). A total of 114 mutants impaired for growth on YNOC agar plates were selected.

3.4 Cloning and disruption of *YAT1* and *YER024w*

The growth defect of the selected mutant strains on YNO plates was complemented by transforming these strains with a *S. cerevisiae* genomic library (ATCC 77162), selecting transformants on glucose containing YND agar plates and selecting

complemented strains through replica-plating on YNOC agar plates. Complementing plasmids were isolated and transformed into *E. coli* DH5α and retransformed into the *S. cerevisiae* mutants to confirm complementation on YNOC agar plates. Genomic inserts were sequenced and the genes *YAT1* and *YER024w* were identified. The *YAT1* gene, with promoter and terminator sequences, was subcloned as a 3962 bp *Hin*dIII-*Eco*RI fragment into the *Hin*dIII and *Eco*RI sites of the plasmid YCplac33. Transformation of the YCplac33-*YAT1* plasmid into the original mutant complemented the growth defect on YNOC agar plates. A portion of the ORF of the *YAT1* gene was deleted by removing the 565 bp *Sac*I fragment by digesting the YCplac33-*YAT1* plasmid with *Sac*I, followed by a religation of the plasmid, resulting in the exclusion of the 565 bp *SacI* fragment from the plasmid. Using this plasmid, the 1.6 kb fragment containing the *LEU2* gene, isolated from plasmid YDp-L, was cloned into the *Bam*HI site of the truncated *YAT1* ORF. A 2.6 kb *Bal*I fragment containing the disruption cassette was isolated and transformed into haploid wild-type FY23 and haploid FY23Δ*cit2*. Leu^+ transformants were selected and the disruption of the chromosomal *YAT1* locus confirmed by PCR through analysing the PCR product obtained from genomic DNA using the primers YAT1F1 and YAT1R1. The *YER024w* gene was subcloned as a 3264 bp *Xba*I-*Hin*dIII fragment into the *Xba*I and *Hin*dIII sites of the plasmid YCplac33. Transformation of the YCplac33-*YER024w* plasmid into the original mutant complemented the growth defect on YNOC agar plates. The YCplac33-*YER024w* plasmid was used and a part of the ORF of *YER024w* gene was deleted by replacing the 871 bp *Pst*I-*Bbr*PI fragment with a 1.6 kb fragment containing the *LEU2* gene, isolated from plasmid YDp-L. A 4.0 kb *Xba*I-*Hin*dIII fragment containing the disruption cassette was isolated and transformed into haploid wild-type FY23 and haploid FY23Δ*cit2*. Leu^+ transformants were selected and the disruption of the chromosomal *YAT1* locus confirmed by PCR obtained from genomic DNA using primers YAT2F1 and YAT2R1.

3.5 CAT assays

The L-carnitine UV-test supplied by Roche Diagnostics (Mannheim, Germany) was adapted for measuring CAT activity. For this purpose, L-carnitine was provided in excess at 400 mg/l in all the samples. Strains were grown in 3% (v/v) ethanol to an OD_{600} of 1.0. A volume of 40 ml of the culture was centrifuged for 5 min at 5000 rpm. The cells were washed once with water and placed into 1.5 ml Eppendorf tubes and centrifuged for 3 min at 5000 rpm. The pellet was suspended in 200 µl of cold 0.05% (v/v) Triton X-100. Glass beads (0.16 g) were added to the suspension and cells were vortexed for 10 min at 8 °C. The suspensions (100 µl) were transferred to new Eppendorf tubes and centrifuged for 3 min at 5000 rpm. The supernatant was used for CAT assays. The amount of NADH consumed during the reaction was measured

spectrophotometrically at 340 nm. The consumption of NADH was linearly correlated to the time of reaction and the gradient of the graph directly correlated with the amount of CAT enzyme added. Samples containing either no L-carnitine or known amounts of CAT enzyme were used as controls. All reactions were carried out in triplicate.

4. Results

4.1 A Δ*cit2* strain is dependent on carnitine for growth on acetate, ethanol, glycerol and oleic acid

In order to phenotypically select for mutants affected specifically for carnitine-dependent metabolic activities, parts of the glyoxylate cycle, which creates a bypass for all known carnitine-dependent activities, have to be blocked. In a strain without a functional *CIT2* gene, mutations in the carnitine shuttle system should indeed result in a growth defect on oleic acid media, but should not affect growth on fermentable carbon sources. To create the appropriate genetic background, the *CIT2* gene, encoding the glyoxylate cycle-specific citrate synthetase which catalyses the condensation of acetyl-CoA with oxaloacetate to form citrate, was disrupted. In this Δ*cit2* mutant strain, the flow of acetyl groups into the glyoxylate cycle is blocked, and the strain will be dependent on the carnitine shuttle to transfer acetyl groups from the peroxisomes to the mitochondria.

The *CIT2* gene was disrupted in the FY23 genetic backgound. As expected, the strain did not present any growth defects on minimal media containing glucose, whether supplemented with carnitine or not. However, when oleic acid was used as sole carbon source, the strain was unable to grow on YNB-based minimal media in the absence of carnitine, but grew as well as the wild-type in the presence of carnitine. In the absence of carnitine, the strain also failed to grow on minimal media containing acetate (YNA), ethanol (YNE) or glycerol (YNG) as sole carbon source (Figure 1 and Table 3). This contradicts previously published data by Kispal et al. (1993), which showed that a PSY142 *S. cerevisiae* with a disrupted *CIT2* locus was able to grow on minimal media containing glycerol as sole carbon source. Other groups have also reported that a Δ*cit2* strain is able to grow on some of the above cited carbon sources (Kim et al., 1986; van Roermund et al., 1999), but these groups use 0.1% yeast extract to complement their growth media. Our selection strategy and phenotype verification was carried out in media containing only carnitine as supplement. Yeast extract indeed contains sufficient amounts of carnitine to complement the carnitine requirements of yeast (van Roermund et al., 1999).

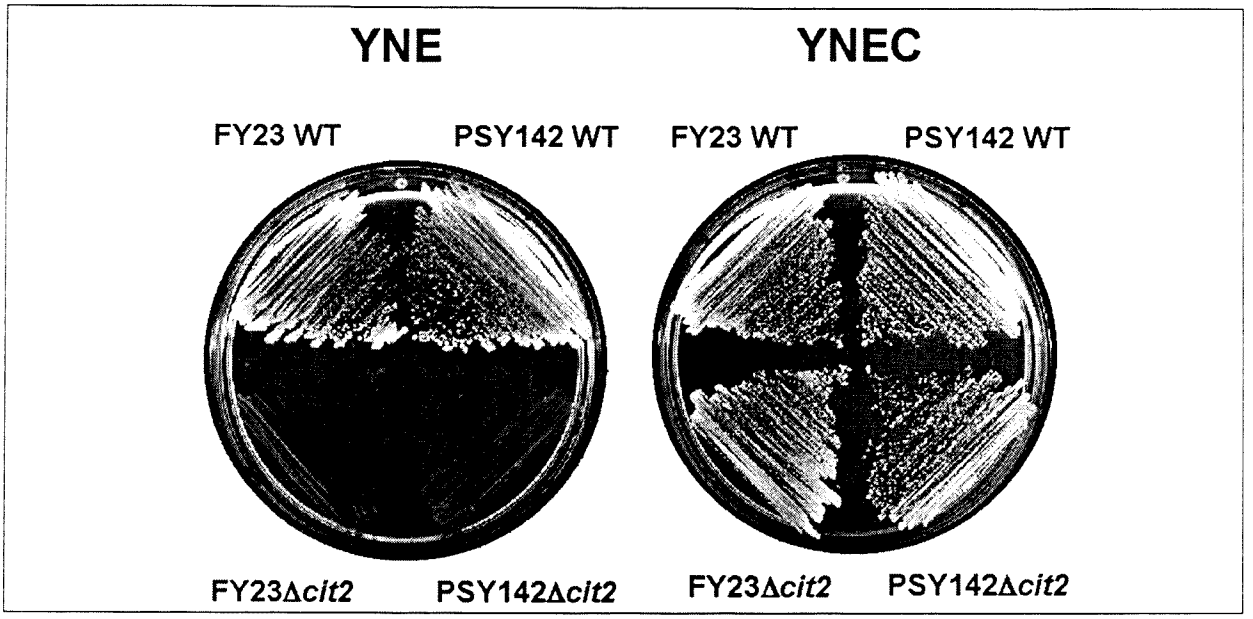

Figure 1. Growth of wild-type (WT) and Δ*cit2* mutant strains on ethanol media with (YNEC) and without L-carnitine (YNE). Plates were incubated at 30° for 10 days.

Table 3. Phenotypes of strains on different carbon sources in the presence and absence of carnitine

Strains	YND	YN/O/A/E/G	YN/OC/AC/EC/GC
FY23	+	+	+
PSY142	+	+	+
FY23Δ*cit2*	+	–	+
PSY142Δ*cit2*	+	–	+
FY23Δ*cit2*Δ*cat2*	+	–	–
FY23Δ*cat2*	+	+	+
FY23Δ*cit2*Δ*yat1*	+	–	–
FY23Δ*yat1*	+	+	+
FY23Δ*cit2*Δ*yat2*	+	–	–
FY23Δ*yat2*	+	+	+

Strains were streaked on YND and incubated at 30°C for 2 days after which it was replica-plated on the different media and incubated for 4-10 days at 30°C (growth +/ no growth –)

The data show that the growth of the Δ*cit2* strains on non-fermentable carbon sources is dependent on extracellular carnitine in the FY23 and PSY142 laboratory strains (Figure 1 and Table 3), which suggests that these strains are unable to synthesise carnitine. This was confirmed through electrospray-masspectrometry (ES-MS) measurements of intracellular carnitine levels in strain FY23 grown on oleic acid which showed that carnitine is only present in the cells when it is supplied in the growth media (data not shown). Taken together with the previously published observation by van Roermund et al. (1999) showing that another laboratory strain, BJ1991, was unable to synthesise carnitine, the data suggest that *S. cerevisiae* is unable to synthesise carnitine.

4.2 Selection of mutants affected in the carnitine shuttle

The mutagenesis of the Δ*cit2* strain resulted in the isolation of a total of 114 mutants defective for growth on oleic acid media supplemented with L-carnitine (YNOC) from a total of 11000 colonies. However, most of these mutants are expected to be affected in genes coding for proteins involved in peroxisome biogenesis, β-oxidation, fatty acid import and other pathways independent of the carnitine shuttle but required for fatty acid utilisation. Such mutants should still be unable to grow on oleic acid even when a functional glyoxylate cycle has been re-established. To select CPAM mutants specifically affected for the carnitine pathway, the 114 strains were individually transformed with a centromeric plasmid containing a functional *CIT2* gene, restoring the glyoxylate cycle in all the mutants. Only mutants with functional peroxisomes and fatty acid utilisation pathways would be able to grow on YNOC medium after transformation with *CIT2*, indicating that the mutations resulting in the defective growth on this medium before transformation with *CIT2* affect genes acting in the carnitine shuttle bypass. A total of six mutants able to grow after retransformation with *CIT2* were isolated (CPAM 1-6).

To verify if the mutants were affected in any of the previously identified carnitine-related genes whose mutation results in related phenotypes, all mutants were transformed with centromeric plasmids containing either *CAT2*, *AGP2* or *CRC1*. One of the mutants, CPAM-3, was complemeted by *CAT2*, while two mutants, CPAM-4 and CPAM5, were complemeted by *CRC1*. These data confirm the high specificity of the selection screen. However, none of the mutants could be complemented by a plasmid carrying *AGP2*.

4.3 The cloning of *YAT1* and *YER024w*

The genes mutated in strains CPAM-2 and CPAM-6 were cloned through functional complementation with a *S. cerevisiae* genomic DNA library. A total of approximately 10 000 transformed colonies were screened for each strain, and four and three complementing plasmids were isolated for CPAM-2 and CPAM-6, respectively. In both cases, one of the isolated plasmids showed a restriction map identical to the chromosomal region containing *CIT2*, and sequencing of these plasmids confirmed that they indeed contained a genomic copy of the gene. This result was expected since functional complementation of the Δ*cit2* genetic background of the mutant strains is sufficient to establish normal growth in all conditions. The other plasmids isolated from each of the mutants contained genomic fragments with partially overlapping restriction maps. The overlapping restriction fragments were subcloned and sequenced. Sequence analysis of plasmids isolated from CPAM-2 showed that the overlapping fragment contained the gene *YAT1*, which codes for the carnitine acetyltransferase presumably associated with the outer mitochondrial membrane

(Schmalix and Bandlow, 1993). This is the first description of a phenotype associated with this gene. CPAM-6, on the other hand, was complemented by a fragment of DNA containing ORF *YER024w*. The predicted protein sequence encoded by *YER024w* displays significant homology with Yat1p, and to a lesser degree with Cat2p. We therefore named the gene *YAT2*.

4.4 Phenotypes of *CAT2*, *YAT1* and *YAT2* deleted strains

To verify the observed phenotypes in null mutants of *CAT2*, *YAT1* and *YAT2*, the genes were disrupted in strains FY23 and FY23Δ*cit2*. The Δ*cat2*, Δ*yat1* and Δ*yat2* strains grew similar to the wild-type strain on all carbon sources tested. However, the double mutant strains Δ*cit2*/Δ*cat2*, Δ*cit2*/Δ*yat1* and Δ*cit2*/Δ*yer024w* did not grow on any of the non-fermentable carbon sources tested (Figure 2 and Table 3), but grew normally on glucose media. The deletion of any of the three genes therefore yielded identical phenotypes to the primary mutants. The data indicate that in the absence of the glyoxylate cycle citrate synthetase, all carnitine acetyltransferases are essential for growth on non-fermentable carbon sources, including oleic acid, acetate, ethanol and glycerol. Transformation of the Δ*cit2*/Δ*cat2*, Δ*cit2*/Δ*yat1* and Δ*cit2*/Δ*yer024w* strains with plasmids carrying *CAT2*, *YAT1* or *YAT2* did not lead to any cross-complementation, indicating that, despite the high sequence similarity, each of the genes encodes for a highly specific activity.

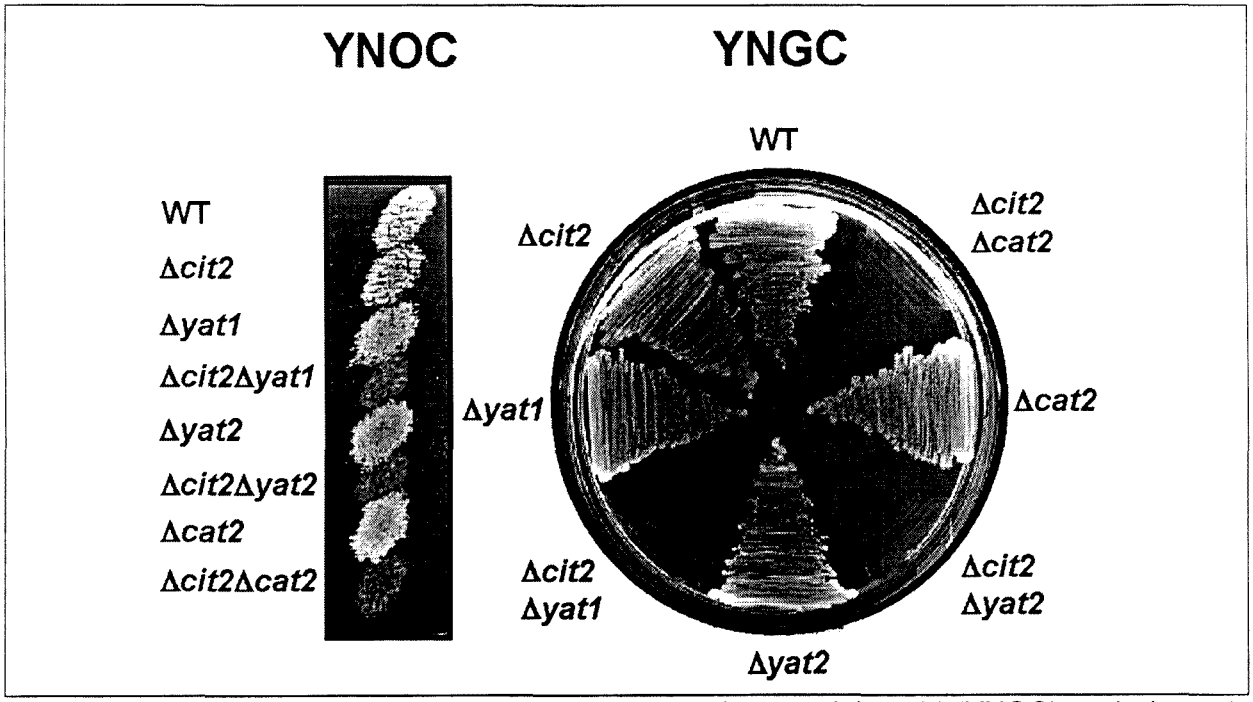

Figure 2. Growth of wild-type (WT) and mutant strains on oleic acid (YNOC) and glycerol (YNGC) media with L-carnitine. Strains were replica-plated on YNOC from a YND master plate and incubated at 30° for 10 days. Strains were streaked on YNGC and incubated at 30° for 8 days.

4.5 Sequence analysis

The predicted amino acid sequence encoded by the *YAT2* ORF displays a significant homology with the two known *S. cerevisiae* carnitine acetyltransferases, Yat1p [29% identity, 45% similarity over 500 amino acids (aa)] and Cat2p (19% identity, 38% similarity over 600 aa), as well as with carnitine acyltransferases from other organisms, where similarities range from 30-40 % (data not shown). Interestingly, the highest similarity between Yat2p and any other protein found in the databases is with FacC, a carnitine acetyltransferase of *Aspergillus nidulans* which has been suggested to represent a cytosolic CAT (Stemple et al., 1998). It is also interesting to note that both Yat1p and Yat2p show a higher degree of sequence similarity with mammalian palmitoyl transferases then with the *S. cerevisiae* Cat2p.

Further analysis of the Yat2p protein sequence indicated the presence of a single acetyltransferase domain, found in a similar position as in Yat1p, while Cat2p has two acetyltransferase domains (Elgersma et al., 1995). No distinct signal sequences, which might target Yat2p to specific cellular compartments in the cell, were detected in the sequence. The identical phenotypes of the three mutants and the sequence homologies strongly suggests that *YAT2* encodes a third CAT in *S. cerevisiae*.

4.6 *YAT2* encodes a CAT contributing 50 % to total CAT activity on ethanol media

In order to verify that *YAT2* indeed encodes a CAT, total CAT activity was measured in a *S. cerevisiae* FY23 wild-type strain and compared with the isogenic Δ*yat2* mutant when grown on ethanol. In these conditions, the mutant showed an approximately 50% reduction in total CAT activity (Figure 3). These data confirm that *YAT2* indeed encodes a functional CAT, which contributes a significant percentage to total CAT activity when cells are grown on ethanol.

5. Discussion

In this study, mutants affected in carnitine-dependent metabolic pathways were isolated. The strategy was based on the finding that there are only two pathways through which peroxisomally produced acetyl-CoA can be utilised for further metabolism. One pathway depends on the carnitine shuttle, while the other depends on the production of glyoxylate cycle intermediates from peroxisomally produced acetyl-CoA and the subsequent transport thereof. By blocking this pathway through the disruption of the *CIT2* gene, we have created a strain that is dependent on the presence of carnitine when acetate, ethanol, glycerol and oleic acid are used as sole carbon sources. These data confirm that laboratory strains of *S. cerevisiae* are unable to neo-synthesise carnitine.

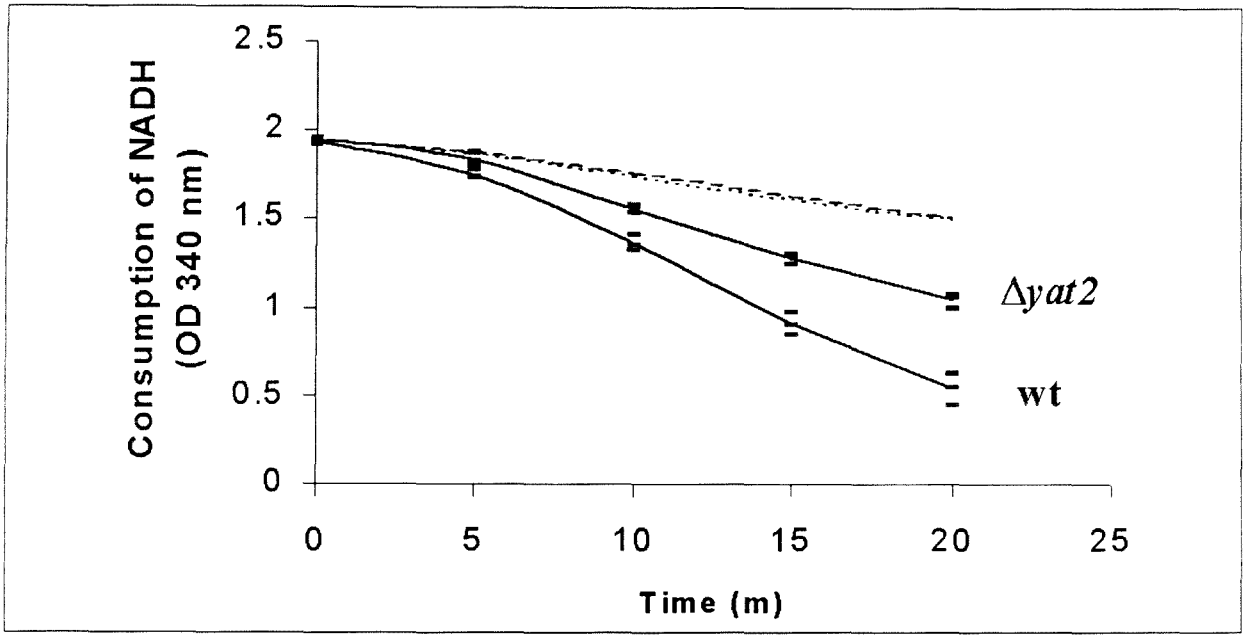

Figure 3. Comparison of CAT activity in wild-type (wt) and Δ*yat2* strains. Cells were grown to OD_{600} of 1 in 3% ethanol media and equal amounts of cells were harvested for mechanical disruption. The amount of NADH consumed during the reaction is directly correlated to the amount of acetylcarnitine formed by CAT. The dotted lines represents the control experiment of the wild type (···) and the Δ*yat2* (---) strains when no carnitine was added to the reaction mixture. The values indicate the average of three independent experiments.

In our study, six mutants affected in carnitine-dependent metabolic pathways were isolated. The genes affected in five of these mutants were identified through complementation. Three mutants were affected in genes encoding CAT enzymes: the *CAT2* gene codes for the peroxisomal and mitochondrial CAT (Kispal et al., 1993; Elgersma et al., 1995), while *YAT1* codes for a CAT associated with the outer mitochondrial membrane (Schmalix and Bandlow, 1993). We show that *YAT2* codes for a third, previously unidentified CAT with significant homology to Yat1p, as well as, to a slightly lesser degree, to Cat2p and CATs of other organisms.

Besides *CAT2* and *YAT1*, two additional genes encoding carnitine-dependent metabolic activities had previously been identified in *S. cerevisiae*: (i) *AGP2*, which codes for a plasma membrane carnitine transporter (van Roermund et al., 1999) and (ii) *CRC1*, coding for the carnitine acetylcarnitine translocase of the inner mitochondrial membrane (Palmieri et al., 1999; van Roermund et al., 1999). All these genes, with the exception of *AGP2*, which also acts as a general amino acid transporter, are specifically involved in the transfer of acetyl groups in and out of organelles *via* the carnitine shuttle. In the selection screen used by van Roermund et al. (1999), ten mutants were selected and could be grouped into only three complementation groups, indicating a rather satisfying level of saturation of the mutant selection procedure. The affected genes were identified as *CAT2*, *AGP2* and *CRC1/CAC*. In our study, we identify, in addition to *CAT2* and *CRC1*, the genes *YAT1* and *YER024w* (*YAT2*). The five analysed mutants therefore yielded four different genes, and none of the mutants was complemented by *AGP2*, suggesting

that mutant selection did not saturate the screen. An explanation for the discrepancy between the data by van Roermund et al. (1999) and the data presented here probably resides in the difference in the two selection strategies. The two strategies differ in the composition of the oleic acid media used for mutant selection, which is either supplemented with carnitine alone (this study) or 0.1% (w/v) yeast extract (van Roermund *et al.*, 1999). While yeast extract contains sufficient amounts of carnitine to meet the requirements of the cells, we also observed that its use in the oleic acid medium (YNOY) resulted in some "background" growth on solid media of all the strains disrupted for *CIT2* together with any of the three carnitine acetyltransferase genes (data not shown), making selection of the mutants more difficult. This could be one of the reasons for the absence of *YAT1* and *YAT2* in the mutant selection screen applied by these authors. Figure 4 shows a schematic representation of the function and localisation of all known proteins involved in carnitine-dependent metabolic activities. It is worthwhile noting that, in addition to the genes and proteins identified to date, there are theoretically two additional, as yet unidentified, proteins required for a functional carnitine shuttle, i.e. the translocases which should be located in the peroxisomal and outer mitochondrial membranes.

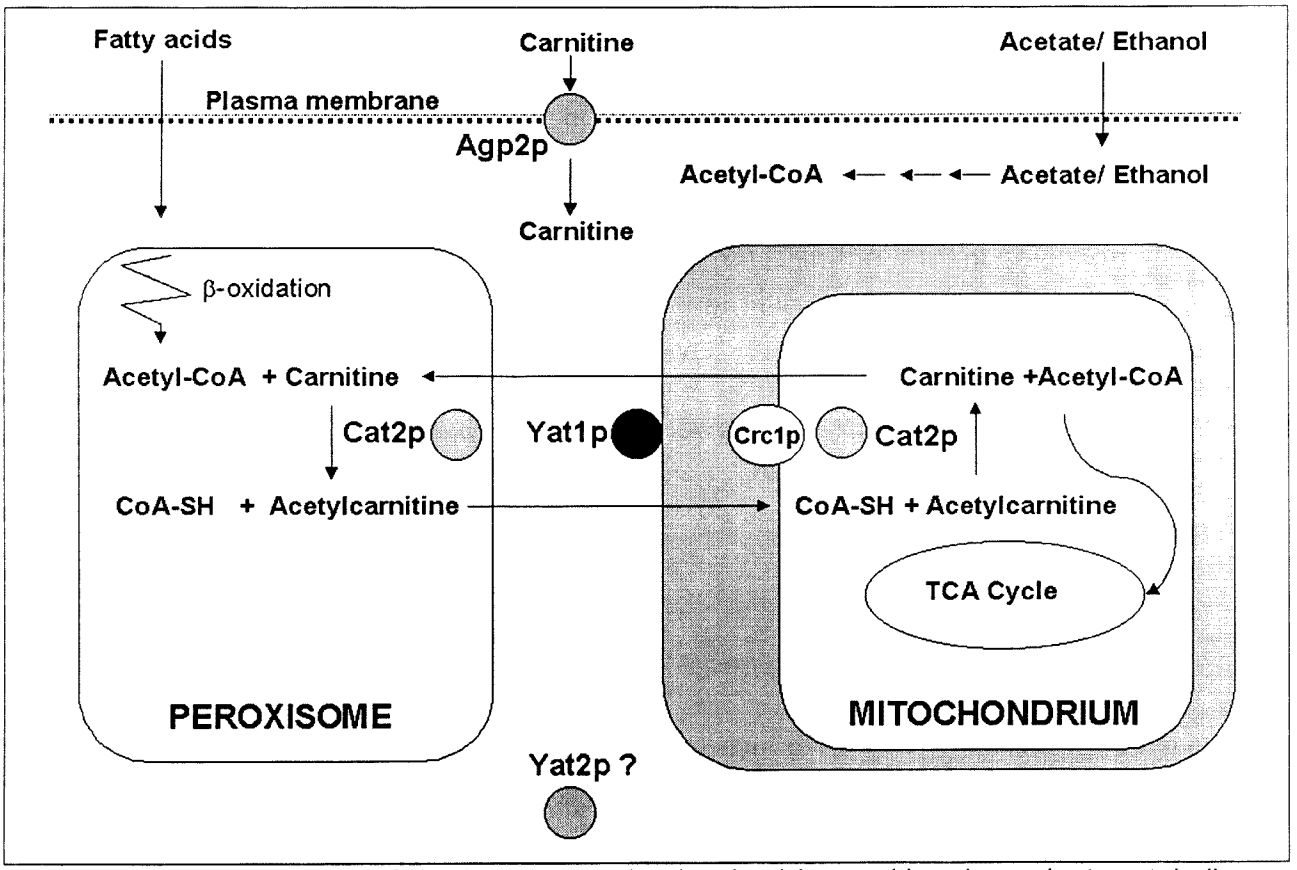

Figure 4. Representation of the known proteins involved in carnitine-dependent metabolic activities. In a Δ*cit2* strain, the cell depends on the carnitine shuttle to transfer acetyl groups to the mitochondria for energy production. Agp2, Cat2p, Crc1p, Yat1p and Yat2p are all essential in a Δ*cit2* strain for growth on fatty acids. The subcellular localisation of Yat2p is not yet known.

The carnitine-dependent phenotype of the Δ*cit2* strain sheds new light on the importance of the glyoxylate cycle. The glyoxylate cycle is indeed required for anabolic purposes on these carbon sources (Kornberg, 1966). It has been proposed that the reason for the survival of the Δ*cit2* strain on these media is that mitochondrially produced citrate can be recruited by the peroxisome for use in the glyoxylate cycle (Kispal et al., 1988; van Roermund et al., 1995). Mutations in the genes coding for the glyoxylate cycle enzymes isocitrate lyase (*ICL1*) and malate synthetase (*MLS1*), on the other hand, renders the strains inviable even on acetate media containing carnitine (McCammon, 1996). These enzymes are responsible for the formation of glyoxylate and succinate from isocitrate and of malate from glyoxylate and acetyl-CoA, respectively. These enzymatic activities are unique to the glyoxylate cycle, and glyoxylate can therefore not be recruited from other metabolic pathways, possibly explaining why strains without Icl1p and Mls1p cannot grow on acetate media.

It is important to take note of the role of carnitine in the metabolism of the yeast when investigating certain genes. In previously published work on the *CIT2* gene, media containing non-fermentable carbon sources including acetate and glycerol have been supplemented with yeast extract (Kim et al., 1986; Kispal et al., 1988). We show that the reported lack of a phenotype of the Δ*cit2* strain in these conditions is due to the presence of carnitine in yeast extract. A discrepancy however exists between data presented by Kispal et al. (1988) indicating that strain PSY142Δ*cit2* grows normally on a minimal glycerol media that does not contain carnitine, and our data, which clearly show that carnitine is required for growth on glycerol media (YNG and YNGC).

The data presented here also shed new light on the importance of CATs in the metabolism of *S. cerevisiae*. We show that all three of these enzymes are essential in a Δ*cit2* strain, and that they play an important role in the metabolism of acetate, ethanol, glycerol and oleic acid. Based on our current knowledge of the carnitine shuttle (Figure 4), it is unclear why three CAT enzymes are essential in all the conditions investigated. It suggests that the physiological importance of carnitine in yeast has been underestimated thus far. Besides the shuttle, carnitine has been suggested to play an important role in the regulation of the availability of free CoA-SH. Considering the highly compartmentalised distribution of CoA, this regulation might require specifically compartmentalised and independently regulated CAT activities. Furthermore, the proper channelling of activated acetyl groups into either anabolic pathways or catabolic energy metabolism is essential during growth on non-fermentable carbon sources, and might require the presence of three independently regulated CATs.

The significant homology of Yat2p with the cytosolic carnitine acetyltransferase of *A. nidulans*, FacC, the absence of distinct signal sequences in the protein and the high percentage of total CAT activity due to Yat2p in ethanol media suggest that this

enzyme could be a cytosolic carnitine acetyltransferase. Cytosolic CAT activity has been detected in *S. cerevisiae* by Kispal et al. (1993), and no CAT responsible for this cytosolic activity has been identified. Further characterisation of the expression of *YAT2* as well as Yat2p's cellular localisation and activity patterns, will cast some light on the role of this protein in the carnitine-dependent metabolic activities in *S. cerevisiae*.

6. Acknowledgements

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CHAPTER 4

RESEARCH RESULTS II

Carnitine biosynthesis in *Neurospora crassa* : Identification of a cDNA coding for ε-*N*-trimethyllysine hydroxylase and its functional expression in *Saccharomyces cerevisiae*

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Carnitine biosynthesis in *Neurospora crassa*: Identification of a cDNA coding for ε-*N*-trimethyllysine hydroxylase and its functional expression in *Saccharomyces cerevisiae*

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1. Abstract

The biosynthesis of L-carnitine in eukaryotic organisms was first elucidated in the ascomycete *Neurospora crassa*. The first step of the pathway is catalysed by ε-*N*-trimethyllysine hydroxylase (TMLH), which converts ε-*N*-trimethyllysine into β-hydroxy-*N*-ε-trimethyllysine in a reaction dependent on α-ketoglutarate, Fe^{2+} and oxygen. Here we report on the cloning of the *N. crassa* TMLH cDNA and its functional expression in *Saccharomyces cerevisiae*. The TMLH cDNA contains an open reading frame (ORF) of 1413 base pairs encoding a predicted polypeptide of 471 amino acids. The Michaelis-Menten constants of the heterologously expressed enzyme were determined for ε-*N*-trimethyllysine, α-ketoglutarate, Fe^{2+} and correspond to 0.33 mM, 133 μM and 46 μM, respectively.

2. Introduction

L-Carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) is required for the transfer of activated acyl groups across intracellular membranes. In most eukaryotic systems, carnitine is an essential component of the mitochondrial carnitine cycle, which is responsible for the transfer of activated long-chain fatty acids into the mitochondria for β-oxidation (Bieber, 1988). In the yeast *Saccharomyces cerevisiae*, carnitine is essential for the transfer of activated acetyl groups from the peroxisome or the cytoplasm to the mitochondrion, since the peroxisomal, and presumably also the mitochondrial membranes are not permeable to acetyl-CoA (van Roermund et al., 1999). Since *S. cerevisiae* does not synthesise carnitine, a strain with a non-functional glyoxylate cycle is dependent on extracellular carnitine for growth on non-

fermentable carbon sources (Swiegers et al., 2001). Most eukaryotes, on the other hand, are able to synthesise carnitine from ε-*N*-trimethyllysine (TML) (Lindstedt and Lindstedt, 1970; Kaufman and Broquist, 1977; Bremer, 1983) TML is provided by the lysosomal hydrolysis of proteins that contain this amino acid as a result of the post-translational modification of lysine residues. In the first step of the carnitine biosynthesis, TML is hydroxylated to β-hydroxy-ε-*N*-trimethyllysine by ε-*N*-trimethyllysine hydroxylase (TMLH, EC 1.14.11.8) (Rebouche and Engel, 1980; Bremer, 1983). Subsequently, β-hydroxy-ε-*N*-trimethyllysine, is cleaved into γ-trimethylamino-butyraldehyde and glycine by β-hydroxy-ε-*N*-trimethyllysine aldolase (Rebouche and Engel, 1980; Bremer, 1983). The aldehyde is then oxidised by γ-trimethylaminobutyraldehyde dehydrogenase to form γ-butyrobetaine (Hulse and Henderson, 1980; Rebouche and Engel, 1980; Bremer, 1983). Finally, γ-butyrobetaine is hydroxylated at the 3-position by γ-butyrobetaine hydroxylase to form L-carnitine (Englard 1979; Rebouche and Engel, 1980; Bremer, 1983).

The identity of the intermediate metabolites of the carnitine biosynthesis pathway was first elucidated in the filamentous fungus *Neurospora crassa*, using isotope-labelling experiments (Kaufman and Broquist, 1977). More recently, enzymes required for the catalysis of three of the four reactions required for carnitine biosynthesis have been characterised at the molecular level, either in rat, mouse or man (Vaz et al., 1998; Galland et al., 1999; Vaz et al., 2000; Vaz et al., 2001). However, none of the *N. crassa* carnitine biosynthesis enzymes has so far been characterised at the molecular level. Here we report the identification of the *N. crassa* cDNA encoding TMLH, the first enzyme of carnitine biosynthesis. TMLH is a non-heme ferrous-iron dioxygenase that requires α-ketoglutarate, Fe^{2+} and molecular oxygen as cofactors (Hulse et al., 1978; Sachan and Broquist, 1980; Sachan and Hoppel, 1980; Stein and Englard, 1982). Based on homology with the recently identified rat TMLH, the putative TMLH ORF was amplified by the polymerase chain reaction (PCR) from a cDNA library, and cloned into an *S. cerevisiae* expression vector. High TMLH activity could be measured in lysates of transformed yeast, allowing for the determination of the kinetic constants of the enzyme for ε-*N*-trimethyllysine, α-ketoglutarate, and Fe^{2+}.

3. Materials and methods

3.1 Cloning and expression of the *N. crassa* TMLH in *S. cerevisiae*

The predicted coding sequence of the hypothetical protein CAC18241 (Genbank™), which is derived from the *N. crassa* genome sequence of DNA linkage group V, Cosmid contig 99H12 position 13250-15449, was used to design nucleotide primers NC-TMLH-F (5'-gatcgaattc **ATG** AGA CCG CAA CGG GTA GGG GCA-3') and NC-

TMLH-R (5'-gatcgaattc **TCA** TTT TCC GCT GGT TTC TTT CCG-3'). These primers were used to amplify the putative open reading frame (ORF) from a *N. crassa* cDNA yeast expression library (Fungal Genetic Stock Center, Kansas, USA) using the PCR technique (Brunelli and Pall, 1993). Using the introduced *Eco*RI sites, the PCR product was cloned downstream of the constitutive *PGK1* promoter of the yeast expression vector pHVXII (Volschenk et al., 1997). The correct orientation of the ORF was confirmed by restriction analysis. The construct was transformed into the yeast strain FY23Δcit2 (Swiegers et al., 2001) using the lithium acetate procedure (Becker and Guarente, 1991). Transformed yeast cells were grown on minimal glucose medium (6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} glucose and amino acids as required at 20 mg L^{-1}). Spheroplasts were prepared using zymolyase according to Franzusoff *et al.* 1991, lysed in a 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl, 1 mM DTT and 1 g/litre Triton X-100 and TMLH activity was measured as described below.

3.2 TMLH assay

The amount of β-hydroxy-ε-*N*-trimethyllysine that was enzymatically produced from ε-*N*-trimethyllysine was determined by HPLC tandem MS as described by Vaz *et al.* 2001. For the measurement of the dependency of TMLH on ε-*N*-trimethyllysine, α-ketoglutarate and Fe^{2+}, only the concentration of these components was varied. To obtain a reliable K_m value, the enzyme sample was diluted so that the substrate consumption was below 10%.

4. Results and Discussion

4.1 Cloning and sequence analysis of *N. crassa* cDNA

A putative *N. crassa* TMLH-encoding gene was identified by database searches based on the homology of the derived protein sequence (CAC18241) to known mammalian TMLHs. The predicted coding sequence of this hypothetical protein was used to design primers overlapping with the putative start and stop codons. Using a *N. crassa* cDNA library as template, a PCR product of 1.6 kb was amplified. Sequencing of the product revealed a smaller than expected ORF of 1413 bp, encoding a protein of 471 amino acids, which corresponds to a calculated molecular mass of 52.6 kDa (EMBL accession no. AJ421151). Alignment with the genomic sequence indicated that the coding sequence consists of eight exons, and that the coding sequence of CAC18241 had been predicted incorrectly, with several intron splice sites having been missed or miss-assigned. This also explains the smaller than expected size of the ORF encoded by the cDNA, with primer NC-TMLH-R hybridising in fact to the sequence 206-226 bp downstream of the newly identified stop codon. The protein sequence derived from the cDNA also aligns better with published TMLH

protein sequences than the previously suggested sequence. The protein indeed has high homology to the three confirmed TMLH proteins identified in rat, mouse and human [13] and also to the last enzyme of carnitine biosynthesis, γ-butyrobetaine hydroxylase (BBH), which is also a dioxygenase. The positional identity to mouse and human TMLH is 33%, and 32% to rat TMLH (Figure 1). The protein contains a unique and unusual 11 residue poly-P region (residues 278-288). This region could separate two protein domains and should have a major effect on the tertiary structure of the enzyme. X-ray crystallisation studies will be pursued to elucidate the effect of this region. No significant homologies to other dioxygenase enzymes were found, indicating that the TMLH and BBH carnitine biosynthesis enzymes form a separate group of highly conserved enzymes.

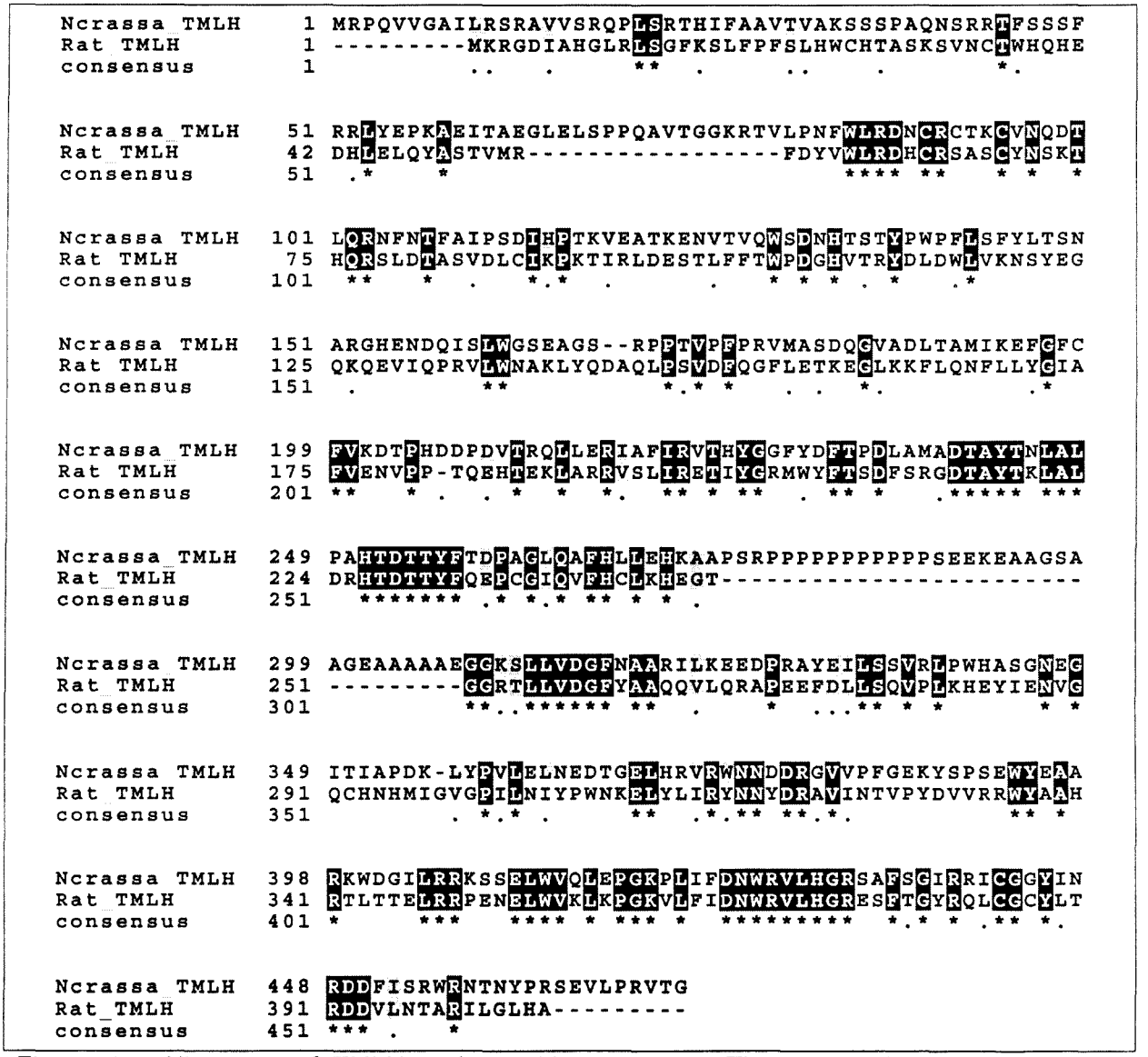

Figure 1. Alignment of TMLH amino acid sequences. The deduced protein sequence encoded by the *N. crassa* TMLH-encoding cDNA (EMBL accession no. AJ421151) was aligned with the sequence of the rat TMLH protein. The *N. crassa* sequence contains an unusual, 11-residue poly-P region (residues 278-288) in the TMLH protein.

4.2 Expression of *N. crassa* TMLH in *S. cerevisiae*

To confirm that the identified sequence indeed encodes a TMLH, the ORF was cloned into the multiple copy expression vector pHVXII between the constitutive *PGK1* promoter and the *PGK1* terminator, and transformed into strain FY23Δcit2. TMLH activity measurement in the homogenate of a yeast transformant containing the putative TMLH cDNA revealed high TMLH activity, whereas the strain transformed with the pHVXII vector without insert showed no activity (Figure 2). The gene was subsequently named *cbs-1* for *c*arnitine *b*iosynthesis. Contrary to rat TMLH, heterologously expressed *N. crassa* TMLH is therefore active in yeast. Rat TMLH is localised in the mitochondria and undergoes post-translational processing, probably after import into the mitochondria (Vaz et al., 2001) whereas *N. crassa* TMLH is a cytosolic enzyme (Kaufman and Broquist, 1977). These differences in post-translational modification and localisation may explain the difference in activity in *S. cerevisiae*.

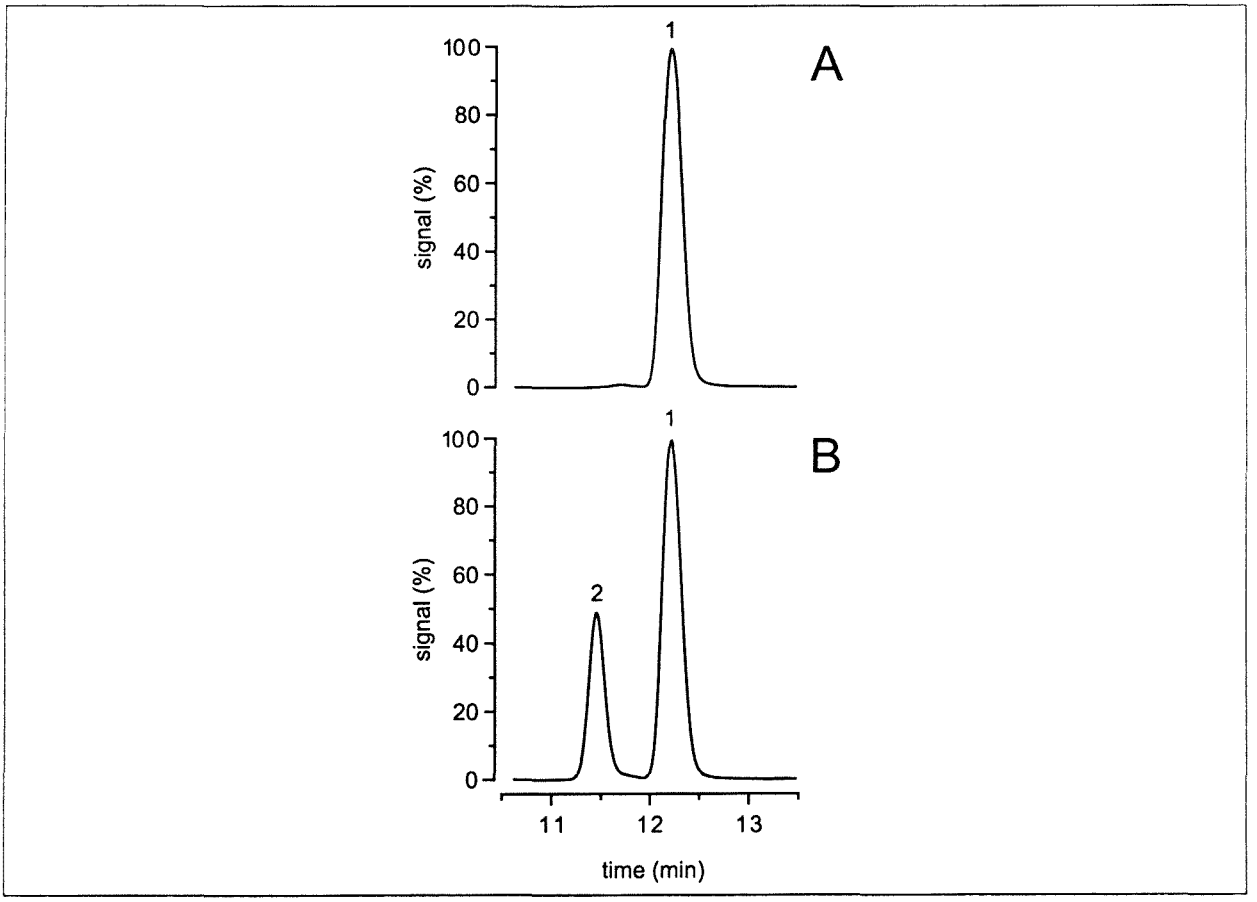

Figure 2. Detection of β-hydroxy-ε-*N*-trimethyllysine by HPLC tandem MS. Levels of ε-*N*-trimethyllysine and β-hydroxy-ε-*N*-trimethyllysine, the substrate and product of TMLH activity, respectively, were measured after 60 minutes incubation in cellular extracts. Peak 1 corresponds to ε-*N*-trimethyllysine and peak 2 to β-hydroxy-ε-*N*-trimethyllysine. (A) Extract from *S. cerevisiae* strain transformed with vector pHVXII without insert. (B) Extract from *S. cerevisiae* strain transformed with vector pHVXII containing inserted TMLH ORF. The trace represents the total ion current of the transitions m/z 247>142 (peak 1, ε-*N*-trimethyllysine) and m/z 263>158 (peak 2, β-hydroxy-ε-*N*-trimethyllysine).

4.3 Characterization of heterologously expressed *N. crassa* TMLH

The K_m values for the substrate ε-*N*-trimethyllysine, as well as the co-factors α-ketoglutarate and Fe^{2+}, were determined using Lineweaver-Burk double-reciprocal plots. The K_m values for the three compounds were found to be 0.33 mM, 133 μM and 46 μM, respectively (Figure 3). The K_m value for ε-*N*-trimethyllysine is significantly lower than the previously reported values of 1.1 mM (Vaz et al., 2001) and 1.6 mM (Sachan and Hoppel, 1980) for rat TMLH. On the other hand, the K_m values for α-ketoglutarate and Fe^{2+}in rat TMLH, which were determined to be 109 μM and 54 μM, respectively, are similar to those reported here (Vaz et al., 2001).

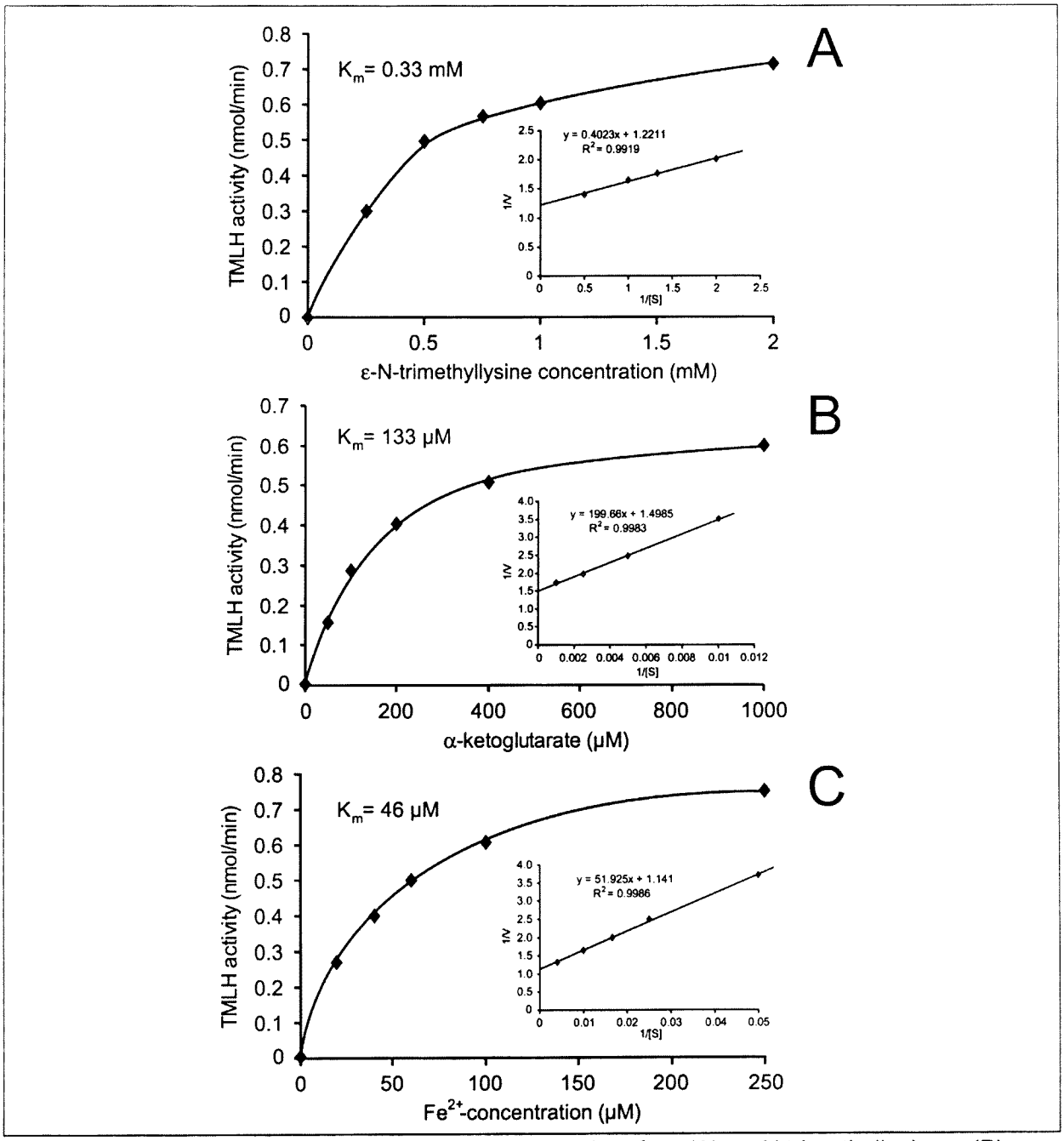

Figure 3. K_m curve and Lineweaver-Burke plot for (A) ε-*N*-trimethyllysine, (B) α-ketogluterate, and (C) Fe^{2+}.

5. Acknowledgements

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CHAPTER 5

RESEARCH RESULTS III

Engineering carnitine biosynthesis in *Saccharomyces cerevisiae*: functional expression of a γ-butyrobetaine hydroxylase from *Neurospora crassa*

This manuscript will be submitted to *Biotechnology and Bioengineering*

Engineering carnitine biosynthesis in *Saccharomyces cerevisiae*: functional expression of a γ-butyrobetaine hydroxylase from *Neurospora crassa*

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1. Abstract

L-Carnitine is a quaternary ammonium compound that plays an essential role in the transfer of activated acyl-residues across intra-cellular membranes. Most eukaryotes can neo-synthesise carnitine, but recent data show that this is not the case in the yeast *Saccharomyces cerevisiae*. The filamentous fungus *Neurospora crassa* was one of the first organisms used to identify the precursor and intermediates of the carnitine biosynthesis pathway. In this organism, the precursor trimethyllysine is converted in a four-step process to carnitine. In the last step of this pathway, γ-butyrobetaine is hydroxylated to form carnitine in a reaction catalysed by γ-butyrobetaine hydroxylase (BBH). A novel plate screen has been developed for the identification of carnitine producing strains of yeast. Using this screen, a genomic fragment encoding the *N. crassa* γ-butyrobetaine hydroxylase was identified and the gene designated *cbs-2*. We show that a yeast expressing *cbs-2* is able to endogenously produce carnitine from γ-butyrobetaine and that expression of this gene is able to rescue the growth defect on non-fermentable carbon sources of the carnitine-dependent Δ*cit2* strain.

2. Introduction

L-Carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) is a quaternary ammonium compound that was first discovered in muscle extracts in 1905 (Bremer, 1983). In 1952, it was shown that the mealworm *Tenebrio molitor* is dependent on carnitine for survival, generating new interest in this molecule, which was named vitamin B_T (Carter et al., 1952). Later investigations showed that most eukaryotic organisms could synthesise L-carnitine from trimethyllysine as a precursor (Vaz and Wanders, 2002). Nevertheless, carnitine deficiencies occur and they are debilitating diseases,

frequently due to genetic mutations (Bonnefont et al., 1999; Lahjouji et al., 2001). Such diseases are characterised by low levels of carnitine in either the serum or in specific tissues. In most cases, patients respond favourably to exogenous dietary supplementation of carnitine (Pons and De Vivo, 1995). In recent times, L-carnitine has also been used for symptomatic treatment in cases of diseases, such as chronic fatigue syndrome, coronary vascular disease, hypoglycemia and muscular myopathies (Kelly, 1998). In addition, carnitine is widely used in nutritional products, such as energy drinks, weight loss supplements and baby formulae (Carter et al., 1995)

In mammalian cells, carnitine is an essential component of the mitochondrial carnitine cycle that is responsible for the transfer of activated long-chain fatty acids into the mitochondria for β-oxidation (Bieber, 1988). In the yeast *Saccharomyces cerevisiae*, on the other hand, β-oxidation occurs solely in the peroxisomes (Kunau et al., 1988). Van Roermund et al. (1995) showed that exogenous carnitine was essential for growth on fatty acids as sole carbon source in the absence of the glyoxylate cycle citrate synthase, Cit2p. Later, Swiegers et al. (2001) showed that in the Δ*cit2* strain, carnitine is essential for growth on all non-fermentable carbon sources. Therefore, *S. cerevisiae* is unable to biosynthesise carnitine endogenously but relies on exogenous carnitine, which is transported into the cell by the general amino acid membrane transporter Agp2p (van Roermund et al., 1999; Swiegers et al., 2001).

Mammals, plants and some fungi are able to biosynthesise carnitine from ε-*N*-trimethyllysine (TML) (Lindstedt and Lindstedt, 1970; Kaufman and Broquist, 1977; Bremer, 1983). In mammals, TML is provided by the lysosomal hydrolysis of proteins that contain this amino acid as a result of the post-translational modification of lysine residues (Bremer, 1983). However, in *Neurospora crassa*, free lysine is trimethylated in the cytosol (Borum and Broquist, 1977). In the first step of carnitine biosynthesis, TML is hydroxylated to β-hydroxy-ε-*N*-trimethyllysine by ε-*N*-trimethyllysine hydroxylase (TMLH; EC1.14.11.8) (Rebouche and Engel, 1980; Bremer, 1983). Subsequently, β-hydroxy-ε-*N*-trimethyllysine is cleaved into γ-trimethylamino-butyraldehyde and glycine by β-hydroxy-ε-*N*-trimethyllysine aldolase (Rebouche and Engel, 1980; Bremer, 1983). The aldehyde is then oxidised by γ-trimethylaminobutyraldehyde dehydrogenase to form γ-butyrobetaine (Hulse and Henderson, 1980; Rebouche and Engel, 1980; Bremer, 1983). Finally, γ-butyrobetaine is hydroxylated at the 3-position by γ-butyrobetaine hydroxylase to form L-carnitine (Figure 1) (BBH; EC 1.14.11.1) (Englard, 1979; Rebouche and Engel, 1980; Bremer, 1983).

The identity of some of the intermediate metabolites of the carnitine biosynthesis pathway was first elucidated in the filamentous fungus *Neurospora crassa*, using isotope-labelling experiments (Kaufman and Broquist, 1977). The genes encoding the enzymes required for the catalysis of three of the four reactions required for

carnitine biosynthesis have been characterised at the molecular level, either in rat, mouse or man (Vaz et al., 1998; Galland et al., 1999, Vaz et al., 1999; Vaz et al., 2000; Vaz et al., 2001). The first gene for carnitine biosynthesis, TMLH, was recently cloned in our laboratory from *N. crassa* cDNA and functionally expressed in *S. cerevisiae*. (Swiegers et al., 2002). Here we report the identification and cloning of a genomic fragment, which we named *cbs-2*, encoding a *N. crassa* γ-butyrobetaine hydroxylase (BBH) and its functional expression in *S. cerevisiae*. A novel plate screen was used to identify genomic fragments of *N. crassa* that could functionally express BBH. We show that the wild type strain transformed with the *cbs-2* gene can use exogenous γ-butyrobetaine from the growth media to biosynthesise carnitine. In addition, the presence of the *cbs-2* gene in the carnitine-dependent strain Δ*cit2* could rescue the growth defect of this strain on non-fermentable carbon sources in the presence of γ-butyrobetaine. This is the first report of an *S. cerevisiae* strain that can biosynthesise carnitine.

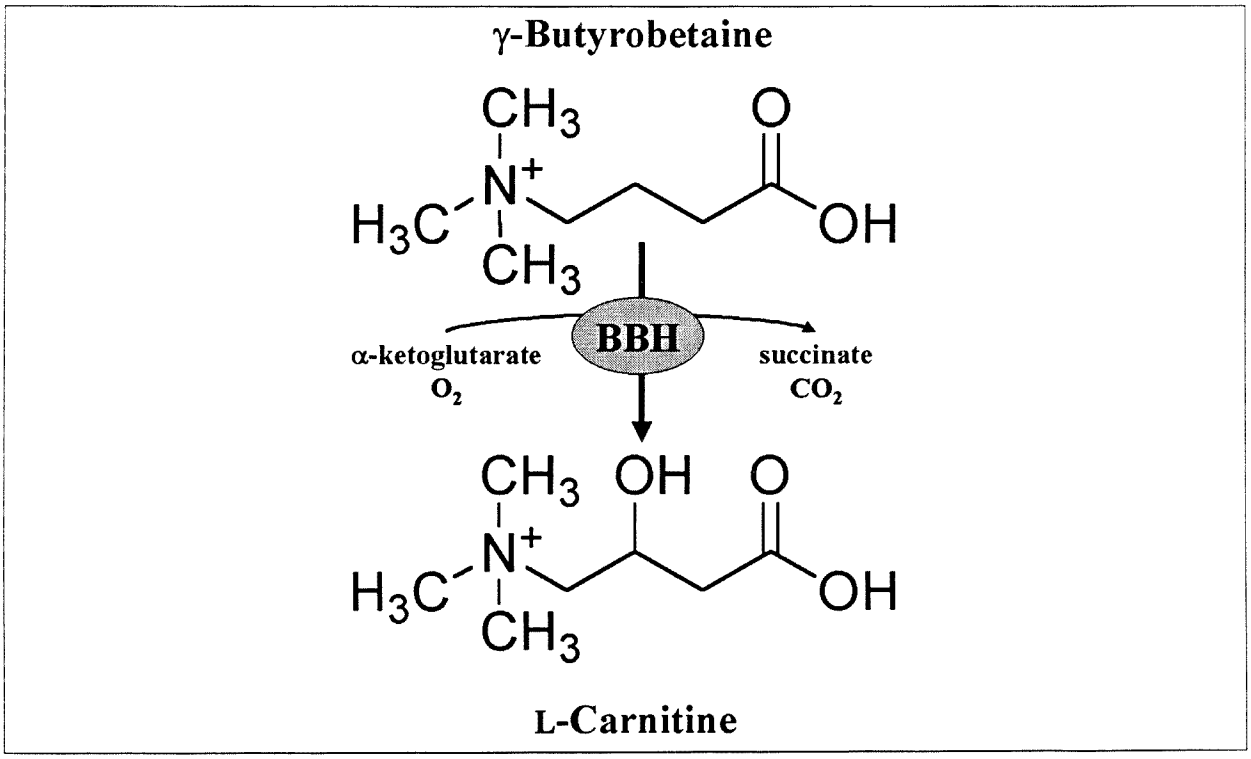

Figure 1. Hydroxylation of γ-butyrobetaine to L-carnitine by γ-butyrobetaine hydroxylase.

3. Materials and methods

3.1 Yeast strains and plasmids

FY23 (*MATa leu2 trp1 ura3*) was used as a wild-type strain (Winston et al., 1995). The FY23Δ*cit2* (*MATa leu2 ura3 cit2*::*TRP1*) was used as the glyoxylate citrate synthase deficient strain (Swiegers et al., 2001). A 2016 bp fragment was cloned

from *N. crassa* genomic DNA using the primers NcBBH-F (5'-GATCAGATCT **ATG** AAA GTC GAC AAG GAA GCC GGC AA-3') and NcBBH-R (5'-GATCAGATCT **TTA** TGC GTT CCA GTT CAC CGT GCC CAA-3') with introduced restriction sites. Genomic DNA was extracted from strain PPRI 3338 (National Collection of Fungi, Agricultural Research Council, Pretoria, South Africa). The fragment was cloned into expression vector pHVXII into the *Bgl*II site under the regulation of the *PGK1* promoter (Volschenk et al., 1997) Sequencing was done using the ABI-Prism sequencer. The *S. cerevisiae* gene *YHL021c* was amplified by PCR from genomic DNA from strain FY23 using the primers YHL-F (5'-GATCGAATTC **ATG** CTA AGA TCA AAT TTA TGC AGA GGA-3') and YHL-R (5'-GATCCTCGAG **TTA** TTT GTA CTG AGG AAA CTT CTC TTC-3') with introduced restriction sites. The fragment was cloned into expression vector pHVXII into the *Bgl*II site under the yeast *PGK1* promoter. Constructs were transformed into the yeast strains using the lithium acetate procedure (Becker and Gaurente, 1991).

3.2 Media and growth conditions

Escherichia coli carrying plasmids were grown in Luria Bertani (LB) broth with 10 mg/l ampicillin. Yeast strains were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose), synthetic glucose medium (6.7 g/l yeast nitrogen base without amino acids, 2% glucose, amino acids as required), synthetic glycerol medium (6.7 g/l yeast nitrogen base without amino acids, 3% glycerol, amino acids as required). Media was prepared using double distilled water.

3.3 Intra-cellular carnitine extraction

Transformants were grown on synthetic glucose medium for two days and then inoculated in 100 ml synthetic glycerol medium with 10 mg/l γ-butyrobetaine and grown for 4 days at 30°C. Cells were harvested by centrifuging 5 min at 5000 rpm and washed with 40 ml double distilled water and harvested again using the same procedure. Cells were resuspended in 1 ml double distilled water, transferred to a 1.5 ml microcentrifuge tube and harvested at 12 000 rpm for 2 min. Wet weight was determined by weighing the cells and the microcentrifuge tube after all the supernatant was removed by pippeting. The cells were resuspended in 0.2 ml double distilled water. The cells were disrupted by adding 0.16 g glass beads and vortexed for 30 min at 8°C. The cells were then vortexed for 10 min at 12 000 rpm and 0.1 ml of the supernatant added to 0.9 ml acetonitrile and stored at -20°C. Before ES-MS analysis, the solution was centrifuged for 10 min at 12 000 rpm to remove all protein precipitates and the supernatant used for analysis.

3.4 HPLC-electrospray mass spectrometry

Mass spectrometry was performed on a Micromass (Manchester, UK) Quattro triple quadropole mass spectrometer fitted with an electrospray ionisation source. Solvent A (acetonitrile/water/formic acid: 30/70/0.05 (v/v/v) was used as a carrier solvent and was supplied to the ionisation source by a LKB/Pharmacia (Sweden) pump. For direct injection of the carnitine and acetylcarnitine standards, the flow rate was 20 µl/min and 5 µl of the standard was injected through a Rheodyne injection valve. The molecular ion ([M+H]+) of carnitine and acetylcarnitine was observed using a capillary voltage of 3.5 kV, source temperature of 80°C and a cone voltage setting of 20 V. To obtain the fragment pattern of carnitine and acetylcarnitine, the molecular ion was dissociated in the fragmentation cell by collision-induced dissociation at an argon pressure of $2.8X10^{-3}$ mbar applying collision energy of 20 eV. The resultant fragments were scanned in the second analyser. Quantitation of carnitine and acetylcarnitine in the incubation samples was accomplished by LCMSMS. A Luna C18 150X2 mm (3µ) column was used for separation, with solvent A as the mobile phase at a flow rate of 100 µl/min delivered by the above mentioned pump. Five microliter of sample was injected by a Waters 747 autosampler. The eluent from the column was directed into the electrospray ionisation source of the mass spectrometer. The capillary voltage, cone voltage, argon pressure and collision energy were as mentioned above. Detection was by multiple reaction monitoring, using the molecular ions of carnitine and acetylcarnitine as precursor ions and the fragments at m/z = 43 and m/z = 85 as product ions, respectively. Chromatographic peaks representing carnitine and acetylcarnitine were integrated and the concentration in the incubation samples were calculated from a dilution range of known concentrations of standard carnitine and acetylcarnitine in distilled water and diluted to a final concentration of 90/10 (v/v): acetonitrile/15 mM Tris.HCl. The calculations were automatically performed by the Quantify program of MassLynx and expressed as ng/ml.

4. Results

4.1 Identification of a *N. crassa* BBH homologue

BBH protein sequences from different organisms are highly homologous to each other and to TMLH protein sequences. BBH and TMLH are part of a family of α-ketoglutarate-dependent, non-haem ferrous iron dioxygenases (Vaz and Wanders, 2002). However, when the BBH and TMLH proteins are compared to other proteins using BLAST searches (NCBI), reduced homology is found, indicating that these enzymes form a separate class of dioxygenases.

Searching the *N. crassa* Genome Database (NCGD) resulted in the identification of 2 putative proteins with high homology to human, rat and mouse BBH protein sequences (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). The first corresponds to the TMLH previously cloned and identified in our laboratory, whereas the second was a novel gene encoding a hypothetical protein (NCU06891.1). The BBH homologous gene predicted by the NCGD consists of 5 predicted exons totalling 3786 bp and translating a hypothetical protein of 1262 aa. This is in strong contrast to the other known BBH proteins of humans, rats, mice and *Pseudomonas*, the length of which varies between 340-380 aa. However, homology to BBH proteins is only found for the protein sequence translated by the last exon (exon 5) as described in the feature map of the hypothetical protein on the NCGD. The other translated exons do not show homology to any known protein.

Using the *N. crassa* genomic DNA, a 2013 bp fragment (encoding a putative protein of 671 aa which includes the entire area of BBH homology), was cloned into a yeast expression vector, pHVXII under regulation of the *PGK1* promotor. Sequencing confirmed that the correct genomic area was cloned. Homology of the 671 aa putative protein to other known BBH proteins from humans, mouse and *Pseudomonas* are shown in Figure 2. However, the 671 aa putative proteins contained a 111 aa N-terminal and 110 aa C-terminal flanking regions without any homology to known BBH proteins. The C-terminal domain contains a six-fold repeat of the sequence "PKVEE". Some 'additional' internal sequences, which contained GGGG repeats, was also present within the BBH homologous area, similar to what was observed for the *N. crassa* TMLH where an 11 residue poly P region and an "AAAAA" are found within the TMLH homologous area (Swiegers et al., 2002).

4.2 Screening of carnitine producing transformants

A large-scale screen was developed to identify microorganisms producing carnitine. The screen is based on the carnitine-dependent Δ*cit2* strain, which, after thorough washing, is plated as a mat on a synthetic agar medium containing a non-fermentable carbon source (e.g. ethanol) and no carnitine. On such plates, growing colonies of microorganisms that biosynthesise carnitine produce a zone due to the complementation of the Δ*cit2* mutant by carnitine. In this way, various microorganisms that produce carnitine were identified, including the yeasts *Yarrowia lipolytica*, *Rhodotorula graminis* and *Candida curvata* (Figure 3B). Endogenous carnitine biosynthesis by these strains was verified through intracellular carnitine/acetylcarnitine measurements using a novel HPLC-electrospray mass spectrometry (ESMS) method that is described in the 'Materials and Methods'.

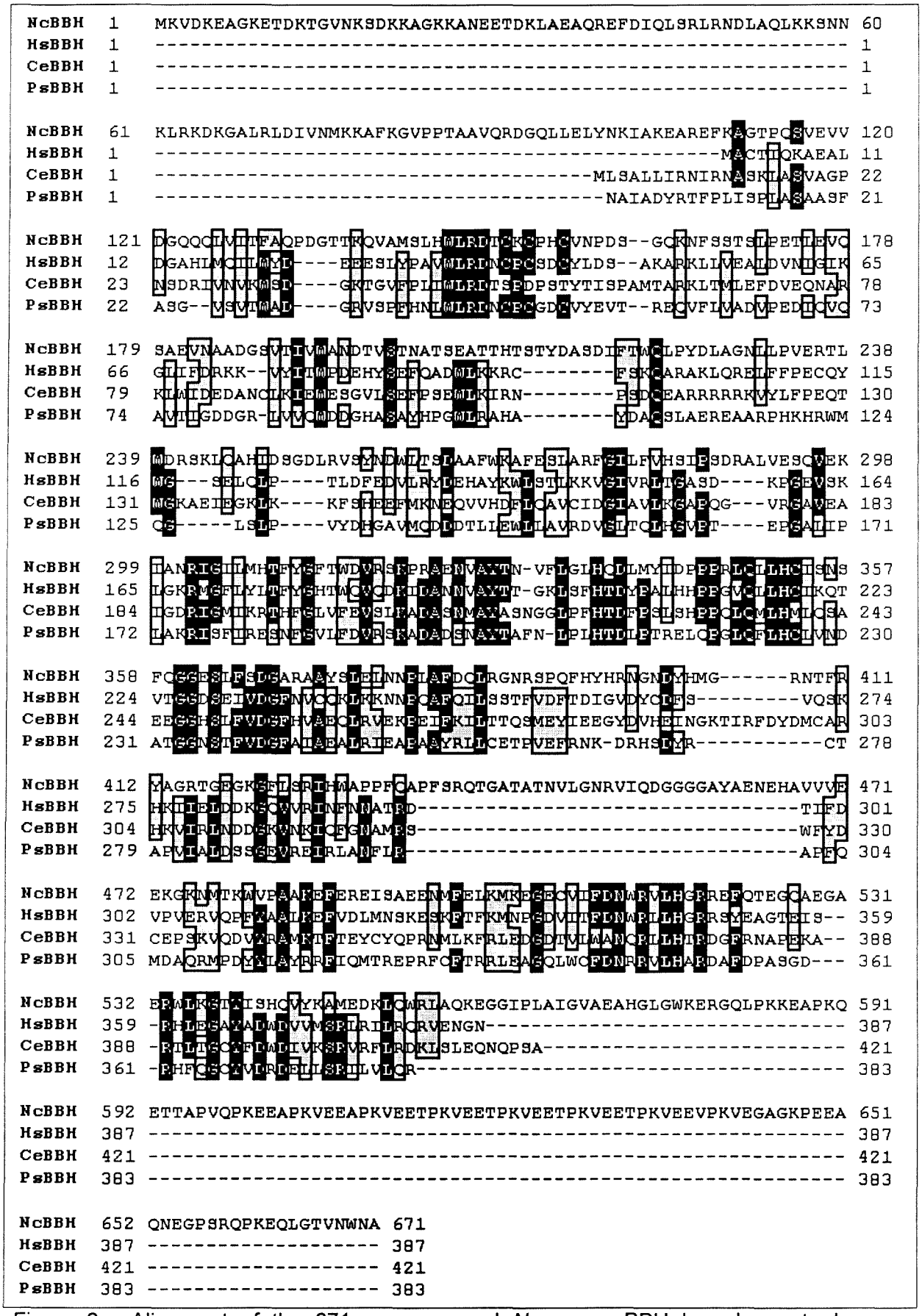

Figure 2. Alignment of the 671 aa expressed *N. crassa* BBH homologue to human, *Caenorhabditis elegans* and *Pseudomonas* BBH proteins.

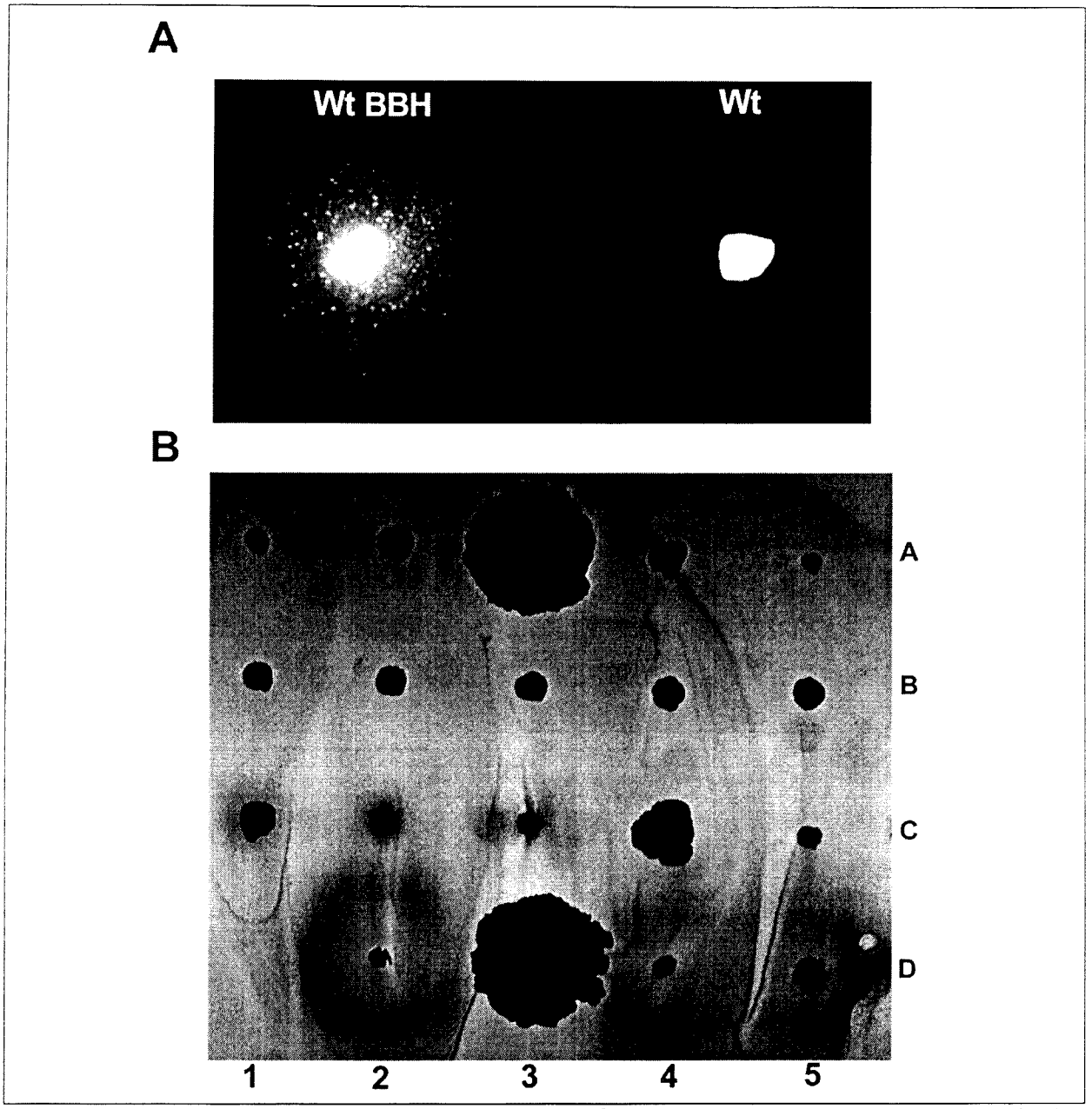

Figure 3. Carnitine large-scale plate screen. (A) Strains were grown on glucose synthetic medium and streaked on a 2% ethanol synthetic medium agar plate with 10 mg/l γ-butyrobetaine and with a thin mat of Δ*cit2* cells grown on synthetic glucose medium, which were washed twice with sterile distilled water before plating. Cells were grown for 10 days at 30°C. The production of L-carnitine by the strain expressing a functional BBH results in the secretion of carnitine, which complements the surrounding Δ*cit2* strains and resulted in the formation of a zone. (B) Carnitine secretion plate assay for identification of endogenous biosynthesis and secretion of carnitine. Yeast were grown on glucose synthetic medium before they were streaked onto a 2% ethanol synthetic medium agar plate with a thin mat Δ*cit2* cells. Cells were grown for 10 days at 30°C. Yeast strains with endogenous L-carnitine biosynthesis and secretion could be identified by the zone formation (D5, D4 and D2). (D5) *Yarrowia lipolytica*; (D4) *Rhodotorula graminis*; (D2) *Candida curvata*. The controls were the *S. cerevisiae* laboratory strain FY23 (A1) and industrial commercial wine strain VIN13 (A2). Other non-zone forming yeast represents a various collection of different genus and species. To improve visual detection of zones, plates were transilluminated with light and photos taken. Biosynthesis of carnitine in zone forming strains was verified using electrospray mass spectrometry as described in the 'Materials and Methods'.

By adapting this large-scale screen, we could screen *S. cerevisiae* strains encoding functional BBH genomic fragments. As in the previous case, washed Δ*cit2* cells are plated on a non-fermentable carbon source but in this instance, γ-butyrobetaine was added to the media to provide the necessary intermediate. Transformed wild type strains forming zones would indicate functionally expressed BBH due to the formation of carnitine from γ-butyrobetaine and its subsequent secretion into the growth medium. Expressing the 2013 bp genomic fragment from the hypothetical protein NCU06891.1, resulted in the formation of a zone (Figure 3A). We concluded that the gene encoded a BBH and therefore named the gene *cbs-2* for "carnitine biosynthesis gene no. 2".

4.3 Complementation of the carnitine-dependent Δ*cit2* strain by BBH

The pHVXII-*cbs-2* construct encoding the 671 aa BBH homologue was transformed into FY23Δ*cit2* in order to see if the transformed strains were able to grow on medium containing the precursor γ-butyrobetaine. The transformants were streaked on synthetic glycerol media with and without γ-butyrobetaine. Strain FY23Δ*cit2* transformed with the *cbs-2* construct, grew in the presence of γ-butyrobetaine, whereas the FY23Δ*cit2* transformed with the vector did not grow on any of the media tested (Figure 4A). Wild type strain FY23 grew normal on both glycerol media tested. The growth effect on glycerol agar plates were also clearly demonstrated on glycerol liquid media where the FY23Δ*cit2* strain transformed with the *cbs-2* construct grew almost like the wild type strain and the FY23Δ*cit2* strain transformed with the vector alone did not grow (Figure 4B). Interestingly, the FY23 wild type transformed with *cbs-2* grew slightly better than the FY23 wild type transformed with vector alone indicating that production of carnitine can be advantageous for the cell or that γ-butyrobetaine may be slightly toxic to the cell. These data suggest that the γ-butyrobetaine in the growth medium is taken up by the Δ*cit2* strain and converted to carnitine endogenously, which allows the carnitine shuttle to function and therefore promote the production of energy and subsequent growth. To confirm this conclusion, carnitine and acetylcarnitine measurements were done using ESMS. Intra-cellular carnitine measurements were made after wild type strains were grown on synthetic medium containing glycerol and synthetic medium containing glycerol with γ-butyrobetaine. No carnitine could be measured in FY23 wild type and FY23 transformed with *cbs-2* in synthetic glycerol medium. When γ-butyrobetaine was added, the FY23 transformed with *cbs-2* gene showed high amounts of carnitine and acetylcarnitine, indicating that carnitine was produced and the carnitine shuttle was active (Figure 5). No carnitine could be measured in the FY23 wild type strain transformed with the vector alone, in the presence of γ-butyrobetaine. A total of 897

ng/gWW of intracellular carnitine and 1151 ng/gWW intracellular acetylcarnitine was measured. *S. cerevisiae* only has carnitine acetyltransferase activity, so acetylcarnitine is the only carnitine ester that can be formed (Swiegers et al., 2001). Carnitine production of *cbs-2* transformed strains could also be confirmed in glucose containing medium.

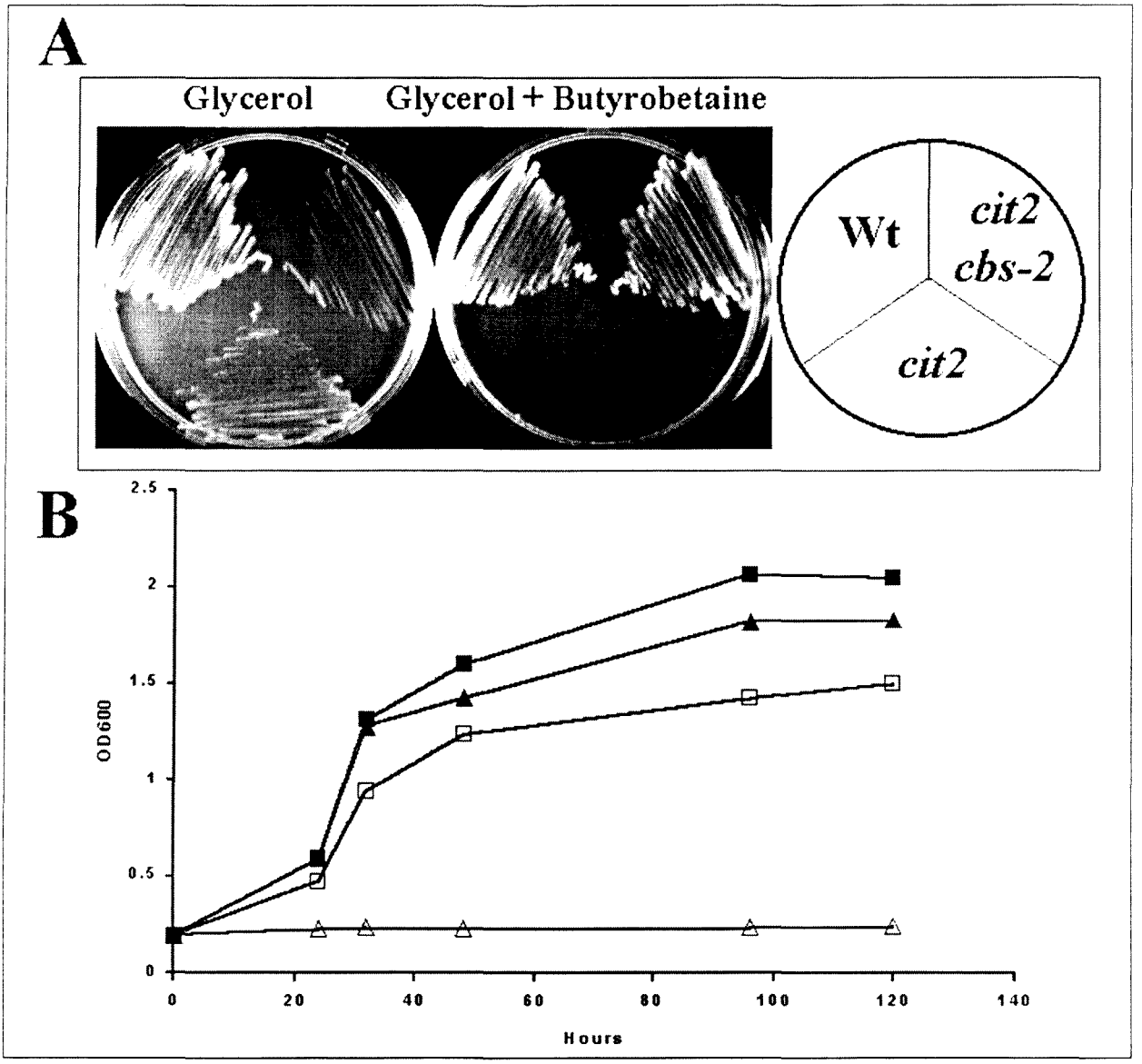

Figure 4. (A) Strains were grown for 4 days at 30°C on synthetic glycerol (3%) medium and synthetic glycerol medium with 10 mg/l γ-butyrobetaine. (B) Growth curves of strains and transformants: FY23 wild type strain (▲); FY23Δ*cit2* strain (Δ); FY23 wild type strain with *cbs-2* (■); FY23Δ*cit2* strain with *cbs-2* (□). Each strain was grown in 100 ml of synthetic glycerol (3%) medium plus 10 mg/l γ-butyrobetaine at 30°C.

4.4 *S. cerevisiae* does not contain genes involved in carnitine biosynthesis

In contrast to most eukaryotic organisms, *S. cerevisiae* cannot endogenously biosynthesise carnitine (van Roermund et al., 1999; Swiegers et al., 2001). However, in this work we show that some other yeasts are able to endogenously biosynthesise this compound *de novo*. To date, all carnitine biosynthesising organisms (including human, rat, mouse, *Caenorhabditis elegans*, *Drosophila melanogaster* and *N. crassa*) have two enzymes with unique function, TMLH and BBH, which are easily identified based on protein homology. However, *S. cerevisiae* has only one putative protein, encoded by *YHL021c*, which has weak homology to the known BBH and TMLH proteins. The Yhl021cp has 22% and 27% identity to human BBH and TMLH proteins, respectively. We cloned *YHL021c* into the yeast expression vector pHVXII and transformed the Δ*cit2* strain. Overexpression of *YHL021c* did not complement the growth defect of the Δ*cit2* strain on non-fermentable carbon sources containing γ-butyrobetaine.

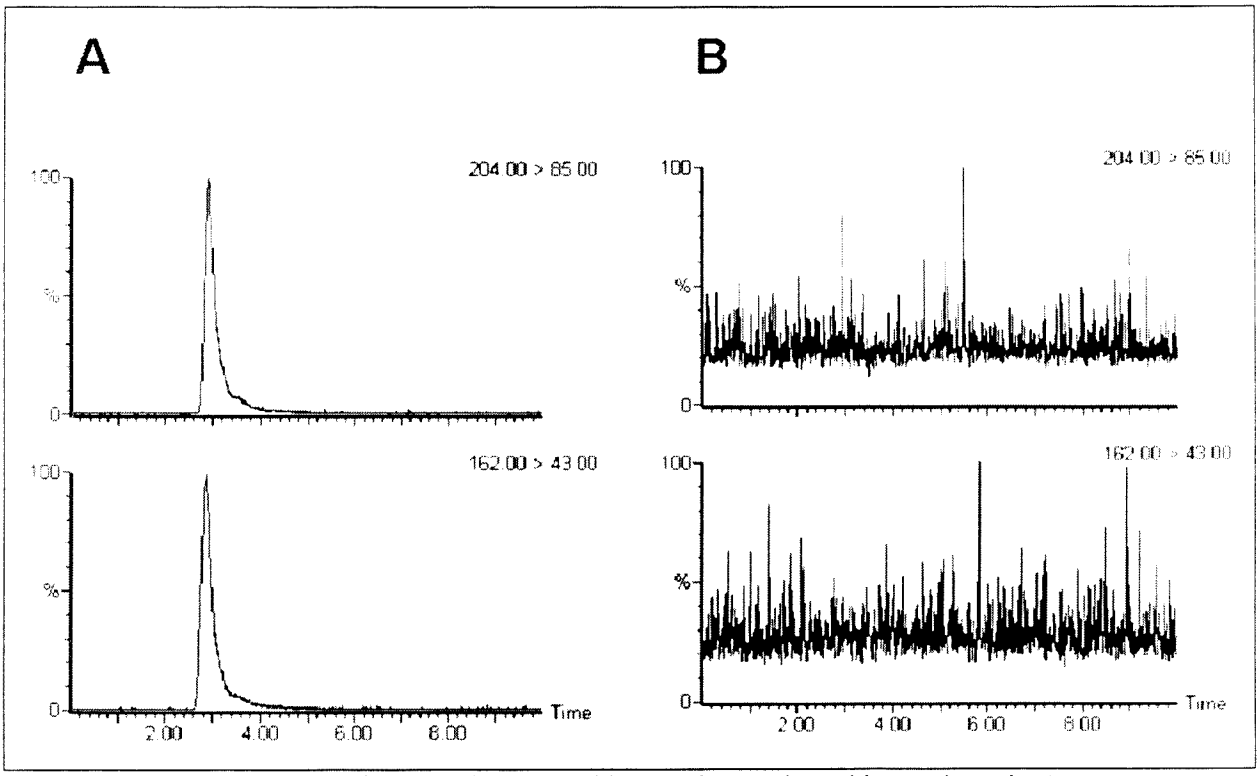

Figure 5. Measurement of intracellular carnitine and acetylcarnitine using electrospray mass spectrometry. (A) FY23 wild type strain with *cbs-2* and (B) FY23 wild type strain was grown on synthetic glycerol (3%) medium with 10 mg/l γ-butyrobetaine for 4 days at 30°C after which cells were harvested and intracellular carnitine and acetylcarnitine levels were determined. Carnitine has a parent ion of 162 and the daughter fragment of 43 was measured. Acetylcarnitine has a parent ion of 204 and a daughter ion of 85 was measured. The level of intracellular carnitine measured for *cbs-2* transformed cells were 897 ng/gWW and acetylcarnitine 1151 ng/gWW.

We also investigated the possibility that expression of both BBH and TMLH could restore biosynthesis of carnitine from trimethyllysine. The two carnitine biosynthesis enzymes involved in the second reaction, 3-hydroxy trimethyllysine aldolase (HTMLA), and third reaction, 4-trimethyl aminobutyraldehyde dehydrogenase (TMABADH), are indeed thought to perform other additional biochemical functions not related to carnitine biosynthesis (Vaz and Wanders 2002). This raises the possibility that similar enzymes may be present in *S. cerevisiae*. The precursor of the carnitine biosynthesis pathway, TML, is present in *S. cerevisiae* (Bieber, 1988). However, co-transformed strains with both *cbs-1* (TMLH) and *cbs-2* (BBH) did not produce any carnitine. Taking all of this together, it is clear that *S. cerevisiae* has no genes involved in carnitine biosynthesis and that most probably all four biosynthesis genes will need to be introduced in order for endogenous carnitine biosynthesis to take place.

5. Discussion

In this study, a *S. cerevisiae* strain was genetically engineered that could biosynthesise carnitine from γ-butyrobetaine. A *N. crassa* genomic fragment was cloned that expressed a functional BBH, which could biosynthesise carnitine from γ-butyrobetaine. In addition, the BBH could suppress the growth defect of the carnitine-dependent Δ*cit2* strain when cells were grown on glycerol synthetic medium containing γ-butyrobetaine.

The hypothetical protein predicted by the NCGB is significantly larger than other known BBH proteins. In future, it will be the focus of our laboratory to establish the role of the upstream and downstream sequences (which was not cloned in this work) of the hypothetical NCU06891.1 gene. However, it might be that due to errors in the sequencing of the *N. crassa* genome, stop codons are present, which will result in a predicted protein that is much smaller. We are currently sequencing the complete NCU06891.1 genomic fragment.

We concluded that to develop a *de novo* carnitine biosynthesising strain of *S. cerevisiae*, probably all four genes encoding biosynthesis enzymes would need to be introduced. In contrast to TMLH and BBH, TMABADH proteins are homologous to a large variety of dehydrogenases, making the cloning of this gene from *N. crassa* through homology searches more difficult. In addition, the HTMLA has not been characterised at the molecular level. This creates particular problems for future work in this field. The use of *N. crassa* biosynthesis mutants might overcome these problems and could lead to the identification of the complete set of genes involved in carnitine biosynthesis.

The use of the large-scale selection screen could be useful to isolate carnitine overproducing mutants through monitoring the zone sizes. It can also be used to

identify the novel carnitine biosynthesis genes from a variety of organisms. The use of carnitine producing strains of *S. cerevisiae* will increase the nutritional value of foods such as bread and beverages such as beer and wine. In addition, carnitine has recently been shown to protect *S. cerevisiae* from stress conditions (Lee et al., 2002). This would be an additional advantage to *S. cerevisiae* strains producing carnitine *de novo*.

6. Acknowledgements

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CHAPTER 6

RESEARCH RESULTS IV

Regulation of respiratory growth by Ras: The glyoxylate cycle mutant, Δ*cit2*, is suppressed by *RAS2*

This manuscript will be submitted to *Journal of Biological Chemistry*

Regulation of respiratory growth by Ras: The glyoxylate cycle mutant, Δ*cit2*, is suppressed by *RAS2*

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1. Abstract

In the yeast *Saccharomyces cerevisiae*, the cytosolic glyoxylate cycle is required for anaplerotic reactions and gluconeogenesis during growth on non-fermentable carbon sources. In these conditions, the glyoxylate cycle and the carnitine shuttle are also the only two pathways through which extra-mitochondrial activated acetyl groups can be further metabolised for energy production in the mitochondria. As a consequence, deletion of the cytosolic glyoxylate cycle citrate synthase, encoded by *CIT2*, causes a severe growth defect on non-fermentable carbon sources in the absence of carnitine. Screening of the Δ*cit2* mutant with an overexpression cDNA library of *Neurospora crassa*, resulted in the isolation of the Ras gene, *ras-1*, as a suppressor. Subsequently, it was shown that the Δ*cit2* mutant could also be suppressed by overexpressing the native *S. cerevisiae RAS2* gene. In wild type strains, overexpression of *RAS2* resulted in enhanced proliferation of cells grown on medium with glycerol as sole carbon source while the constitutively active $RAS2^{val19}$ allele caused a growth defect. However, in the Δ*tpk1* strain, this proliferation effect was blocked. In this study, it was shown that overexpression of *RAS2* nevertheless activates the cAMP/PKA pathway but that activation is not as severe as in the case of the $RAS2^{val19}$ allele. In strains grown on medium containing glycerol as sole carbon source, overexpression of *RAS2* upregulated mitochondrial citrate synthase activity while in the Δ*ras2* mutant, activity was downregulated. We propose that *RAS2* overexpression results in a combination of general upregulation of the mitochondrial machinery and an increase in mitochondrial citrate/citrate synthases, which together, complement the metabolic requirements of the Δ*cit2* mutant.

2. Introduction

In *Saccharomyces cerevisiae*, Ras signals via the cAMP/protein kinase A (PKA) pathway to regulate cellular metabolism in response to the type of carbon source available for utilisation (Broach and Deschenes, 1990; Thevelein, 1994). Two Ras

proteins are present in yeast and are encoded by *RAS1* and *RAS2* (DeFeo-Jones et al., 1983; Powers et al., 1984; Dhar et al., 1984; Toda et al., 1985). Regulatory signals are transmitted by Ras by shuttling between the inactive GDP-bound form and the active GTP-bound form through the activity of the guanine nucleotide exchange factor, Cdc25p, and the GTPase activating proteins Ira1p and Ira2p (Tanaka et al., 1989; Tanaka et al., 1990a; Tanaka et al., 1990b; Jones et al., 1991). The GTP-bound Ras protein stimulates adenylyl cyclase, Cyr1p, which synthesises cyclic AMP, thereby increasing intracellular concentrations of cAMP (Toda et al., 1985; De Venditis et al., 1986; Field et al., 1988). High cAMP concentrations stimulates the binding of cAMP to the PKA regulatory subunit, Bcy1p, thereby releasing the catalytic subunits, Tpk1p, Tpk2p and Tpk3p, which subsequently phosphorylate a variety of proteins involved in cellular metabolism and regulation (Matsumoto et al., 1982; Toda et al., 1987b; Broach and Deschenes, 1990; Thevelein, 1994; Robertson et al., 2000). Intracellular cAMP is degraded by cyclic nucleotide phosphodiesterases, Pde1p and Pde2p (Sass et al., 1986; Nikawa et al., 1987).

The effect of Ras activation on levels of cAMP appears most apparent when strains growing on non-fermentable carbon sources are switched to glucose media (Mbonyi et al, 1990). In these conditions a rapid and significant increase in cAMP levels are observed, as much as a 50-fold increase after 1-2 min. However, cAMP levels rapidly decline and, after a relatively short time, near basal levels are reached (Nikawa et al., 1987). After the cAMP spike, the basal levels of cAMP required for continuous growth on glucose is slightly higher than the basal level of cAMP required for growth on non-fermentable carbon sources. Indeed, the level of cAMP must drop in order for the cell to adapt its metabolism from fermentative growth to respiratory growth (Russel et al., 1993).

The absence of Ras2p downregulates the Ras/cAMP/PKA pathway (decrease of intracellular cAMP concentrations) and this results in a severe growth defect on non-fermentable carbon sources (Fraenkel, 1985; Tatchell et al., 1985; Toda et al., 1985). On fermentable carbon sources e.g. glucose, the absence of Ras2p or Ras1p has no effect on growth, however, when they are both absent, the strain is not viable (Kataoka et al., 1984; Tatchell et al., 1984). Hyperactivation of the Ras/cAMP/PKA pathway (increase of intracellular cAMP concentrations), through the presence of the constitutively active $RAS2^{val19}$ allele, also results in a severe growth defect on non-fermentable carbon sources (Kataoka et al., 1984; Toda et al., 1985; Nikawa et al., 1987). In addition, hyperactivated Ras strains show distinctive phenotypes that include reduction in glycogen and trehalose levels, heatshock sensitivity, nutrient-starvation sensitivity, pronounced pseudohyphal differentiation and invasive growth (Toda 1987a, Engleberg 1994; Pan and Heitman; 1999; Stanhill et al., 1999). The same phenotypes, including a growth defect on glycerol, are observed in strains without the PKA regulatory unit, Bcy1p, where the Tpk's are permanently liberated

and therefore constitutively active (Toda et al., 1985; Toda et al., 1987a). Thus, it is clear that Ras2p/cAMP/PKA pathway is an important regulator of respiratory growth on non-fermentable carbon sources. It has also been shown on the non-fermentable carbon source lactate, that hyperactivation of the cAMP/PKA pathway (e.g. *RAS2*^{val19}) or constitutive activation of PKA (e.g. Δ*bcy1*) results in an increase in mitochondrial enzyme content, while a loss of Ras activity (e.g. Δ*ras2*), results in a decrease in mitochondrial enzyme content (Dejean et al., 2002). The specific molecular targets involved in the mitochondrial upregulation remain to be identified.

The glyoxylate cycle is an essential metabolic process required for growth on non-fermentable carbon sources. The cycle is a modified version of the TCA cycle, incorporating two acetyl-CoA units per cycle and releasing succinate (Kornberg, 1966). The succinate produced by the glyoxylate cycle is transferred to the mitochondria in order to supply the TCA cycle with C_4 intermediates (van Roermund et al., 1995). The carnitine shuttle also supplies the TCA cycle with carbon units. In this shuttle, acetyl-CoA combines with carnitine to form acetylcarnitine, which is transported into the mitochondria where the acetylgroup is released to form acetyl-CoA, which can then enter the TCA cycle (Bremer, 1983). Because of the impermeability of the mitochondrial membrane to acetyl-CoA, the glyoxylate cycle and the carnitine shuttle are the only two pathways through which the carbon groups of cytosolic and peroxisomal acetyl-CoA can be transferred to the mitochondria (van Roermund et al., 1995; Swiegers et al., 2001). In the absence of carnitine (and therefore the carnitine shuttle), the glyoxylate cycle citrate synthase mutant, Δ*cit2*, has a severe growth defect on glycerol (Swiegers et al., 2001). In this mutant, the inflow of acetyl-CoA into the glyoxylate cycle is blocked. However, because the glyoxylate cycle is required for gluconeogenesis and anaplerotic reactions, growth of the Δ*cit2* mutant on non-fermentable carbon sources when supplemented with carnitine implies that the glyoxylate cycle is still functional. Therefore, it has been proposed that in this mutant, citrate is recruited from the mitochondria in order to keep the glyoxylate cycle functioning (van Roermund et al., 1995).

The genomes of filamentous fungi are more complex than the relatively simple genome of *S. cerevisiae*. In addition, the metabolic pathways of filamentous fungi and the regulation thereof differ from that of *S. cerevisiae*, in which glucose fermentation is mostly favoured. The *Neurospora crassa* genome has recently been sequenced and it revealed the interesting complexity of this organism's genetic make-up (Galagan et al., 2003). Of all the predicted proteins, more than 40% have no clear homologues in other eukaryotic organisms and of the more than 1400 genes with high homology to plants and animals, over 500 genes have no homology to genes in *S. cerevisiae* (Hynes, 2003). It would therefore be interesting to see if some of these genes would be able to suppress the *S. cerevisiae* metabolic Δ*cit2* mutant when grown on synthetic medium with glycerol as sole carbon source.

Here we present data showing that both *N. crassa ras-1* and *RAS2* could suppress Δ*cit2* strains when grown on synthetic media with glycerol as carbon source. We show that overexpression of *RAS2* and *ras-1* enhances the ability of yeast to proliferate on glycerol medium in general. Interestingly, the *RAS2* proliferation effect is more pronounced in the Δ*cit2* strain, suggesting communication between the mitochondria and the glyoxylate cycle. We confirm the role of the PKA pathway in this process by showing that in the absence of Tpk1p, the growth enhancement caused by *RAS2* overexpression is blocked. Differences in glycogen levels and flocculation phenotypes suggest that the overexpression of *RAS2* activates the cAMP/PKA pathway but less severely than in the case of the $RAS2^{val19}$ allele. Furthermore, our data show that overexpression of *RAS2* increases and Δ*ras2* decreases mitochondrial citrate synthase activity of cells grown on glycerol. We propose that a combination of the upregulation of mitochondrial capacity and increase in mitochondrial citrate/citrate synthases, complements the metabolic needs of the Δ*cit2* mutant. Therefore, the suppression does not act directly on the glyoxylate cycle but reflects the indirect effects resulting from general mitochondrial upregulation.

3. Experimental procedures

3.1 Yeast strains and plasmids

FY23 (*MATa leu2 trp1 ura3*) was used as a wild-type strain while the FY23Δ*cit2* (*MATa leu2 ura3 cit2::TRP1*) was used as the glyoxylate citrate synhase deficient strain (Winston et al., 1995; Swiegers et al., 2001). The *RAS2* gene was cloned by PCR from plasmid YCP50-*RAS2* using the primers RAS2F(EcoRI) 5'-GATCGAATTC **ATG** CCT TTG AAC AAG TCG AAC A-3' and RAS2R(XhoI) 5'-GATCCTCGAG **TTA** ACT TAT AAT ACA ACA GCC AC-3' with introduced restriction sites (underlined). The gene was subcloned into pGEM-T-easy (Promega) and cloned into the *Eco*RI/*Xho*I sites of expression vector pHVXII between the *PGK1* promoter and terminator (Volschenk et al., 1997). Transformation of yeast was done using the lithium acetate procedure (Becker and Gaurente, 1991). The $RAS2^{val19}$ allele was supplied by David Engelberg (plasmid B2562). The pYPGE15 *ras-1* plasmid was isolated from PG15 cDNA library (Fungal Genetic Stock Center, Kansas City, KS, USA) (Brunelli and Pall, 1993). This library is based on the pYPGE15 plasmid; 2 micron, *URA3* and the cDNA's were cloned under regulation of the constitutive *PGK1* yeast promoter. The FY23Δ*ras2* strains was prepared by transforming the PCR product of the disruption cassette of strain BY4742Δ*ras2* (Euroscarf) using the primers RAS2-Fp AGT GGG TGG TGT GGC TAA TC and RAS2-Rp CAT CGT CGT CTT CCT CG. Other strains used were BY4742wt, BY4742Δ*tpk1*, BY4742Δ*tpk2* and BY4742Δ*tpk3* (Euroscarf) and disruptions were verified using PCR.

3.2 Media and growth conditions

Yeast were grown in rich 2% glucose medium (YPD), synthetic glucose medium (SCD); 6,7 g/l yeast nitrogen base without amino acids (Difco) and 2% glucose. Synthetic glycerol medium (SCG) contained 6.7 g/l yeast nitrogen base without amino acids (Difco) and 3% glycerol. Amino acids were supplied according to the requirement of each strain.

3.3 cDNA library screen

A *N. crassa* cDNA yeast expression library (Fungal Genetic Stock Center, Kansas City, KS, USA) was used to transform the FY23Δ*cit2* strain using the lithium acetate method (Becker and Guarente, 1991). About 40 000 transformants were replica plated on YNG medium. Plates were incubated for two weeks at 30°C. Clones that grew were isolated and the plamids isolated. Isolated plasmids were retransformed into the FY23Δ*cit2* strain to confirm the phenotype. Sequencing was done using the ABI-Prism automated sequencer.

3.4 Citrate synthase and citrate assay

Strains were grown in selective media and 4 ml of the culture was harvested and the supernatant decanted. The culture was centrifuged and the remaining supernatant removed by pipetting. The cells were resuspended in 200 µl ice cold Triton-X-100 (0.05%); Tris-HCl 0.1 M solution and 150 µl glass beads were added. The suspension was vortexed rigorously at 8°C for 15 min and 800 µl ice cold water was added. A volume of 25-50 µl was used for enzyme analysis. Citrate synthase (EC 4.1.3.7) activity was determined by monitoring at 412 nm the oxidation of coenzyme A (produced by citrate synthase activity) by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as a function of time using a photometer (Srere, 1969). The enzyme activity was calculated using an extinction coefficient of 13600 M^{-1} cm^{-1} at 412 nm. One citrate synthase unit was equal to 1 µmole of DTNB reduced per minute per mg wet weight

For intracellular citrate determination strains were grown on selective SCD medium for 48 hours and 40 ml harvested through centrifugation for 5 min at 5 000 rpm. Cells were washed with 10 ml distilled water, harvested and resuspended in 1 ml distilled water to be transferred to a microcentrifuge tube. After centrifuging for 1 min at 12 000 rpm and removal of the supernatant, 0.5 g of glass beads were added and 0.3 ml Triton-X-100. Rigorous vortexing was applied for 30 min at 8°C. The microcentrifuge tubes with cell suspension were centrifuged 10 min, 12 000 rpm

at 4°C to remove the debries. The supernatant was used to assess the citrate content using the Citric Acid Enzymatic UV test kit (Roche).

3.5 cAMP assay

The BiotrakTM cAMP competitive enzyme-immunoassay system was used to determine intracellular levels of cAMP (Amersham Pharmacia Biotech). Strains were grown in selective media and a total of 10-40 ml of cells were harvested depending on the growth stage (Figure 3C). Cells were resuspended in 1 ml water and transferred to a microcentrifuge tube. The cells were harvested and the wet weight determined. The cells were resuspended in 300 µl lysis buffer 1B (Amersham) and 150 µl glass beads added. The suspension was vortexed for 30 min at 8°C and then spun down 10 min at 4°C. The supernatant (100 µl per sample) was used for analysis.

4. Results

4.1 Cloning of heterologous suppressors of Δ*cit2*

We used the *N. crassa* cDNA yeast expression library to screen for suppressors of the Δ*cit2* mutation in *S. cerevisiae* (Brunelli and Pall, 1993). The library was transformed into the Δ*cit2* mutant and transformants selected on SCD media. Approximately 40 000 transformants were replica-plated onto SCG plates without carnitine. After two weeks, four growing colonies were isolated and the plasmids were retrieved and sequenced. Two plasmids contained cDNA that was identified as the *ras-1* gene, the *N. crassa* homologue to yeast *RAS1* and *RAS2* (NCBI accession no. X53533 protein id. CAA37612.1) (Atschuler et al., 1990). The other two suppressors were identified as coding for an ATPase but was not investigated further. No citrate synthase homologues were isolated indicating that the library was not saturated. The *ras-1* gene codes for a protein of 213 aa. The translated protein has 59% and 57% identity to yeast *RAS1* and *RAS2* respectively. Retransformation of the *ras-1* clone confirmed the suppression of Δ*cit2* (Figure 1). Transformation of the plasmid into a *S. cerevisiae* Δ*ras2* strains suppressed the growth defect of this mutant on non-fermentable carbon sources indicating the functionality of the *N. crassa ras-1* gene in *S. cerevisiae* (data not shown). The interchangability of Ras proteins between organisms is well known and this is the first time it has been shown for *N. crassa* (Kataoka et al., 1985; Parrini et al., 1996).

4.2 Yeast *RAS2* suppresses the Δ*cit2* growth defect

The identification of a Ras gene as a suppressor of Δ*cit2* was a surprising but interesting find. In order to assess if this suppression was exclusively linked to the *N. crassa* Ras, or if the *S. cerevisiae* native Ras genes could also suppress the Δ*cit2* mutant, the *RAS2* gene was cloned into the multiple-copy expression vector pHVXII under the regulation of the *PGK1* promoter. The FY23Δ*cit2* strain was transformed with pHVXII-*RAS2*, grown under selective conditions in SCD medium, serially diluted and spotted on 3% SCG agar plates. After 8 days a clear suppression of the growth defect of the Δ*cit2* mutant was observed in pHVXII-*RAS2* transformed strains, similar to the suppression of Δ*cit2* by *N. crassa ras-1* overexpression (Figure 1). However, the constitutively active $RAS2^{val19}$ could not suppress the Δ*cit2* mutant.

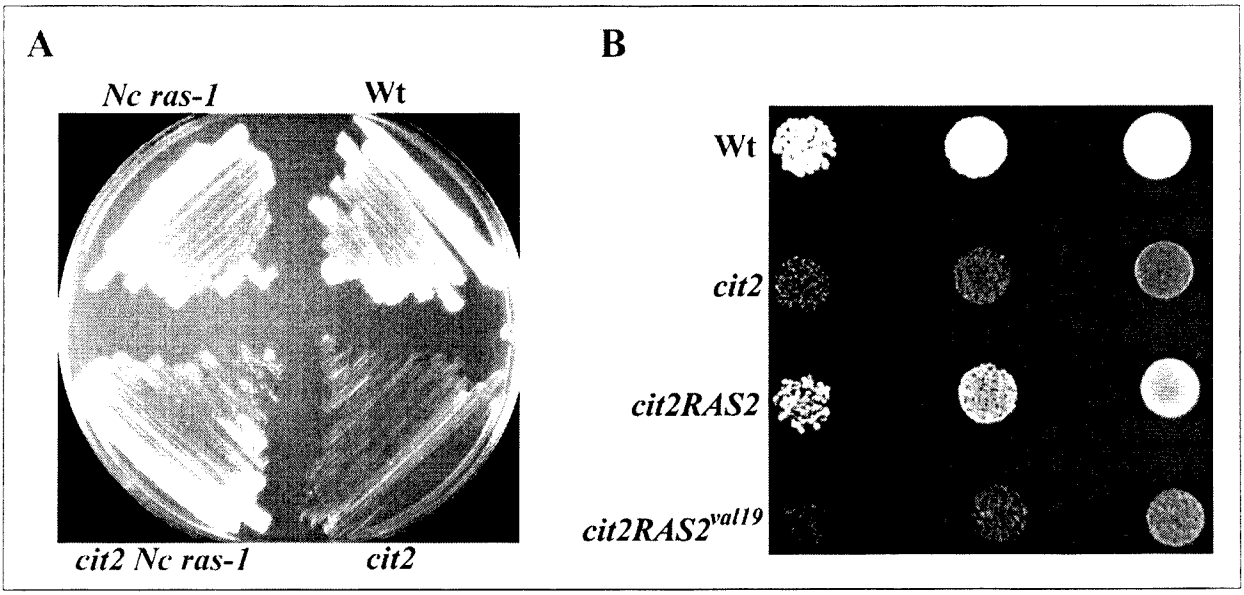


Figure 1. Suppression of Δ*cit2* by Ras on glycerol. (**A**) Suppression of the Δ*cit2* phenotype by *Neurospora crassa ras-1*. Strains were grown on SCD to avoid carnitine carry-over from rich media and then streaked on SCG and grown for 14 days at 30°C. (**B**) Suppression of the Δ*cit2* phenotype by *RAS2*. Strains were grown on SCD solid media and then for 2 days on SCD liquid media to stationary phase. Strains were serially diluted to equal cell counts and then spotted on SCG media and grown for 8 days at 30°C.

The suppression of the Δ*cit2* phenotype by overexpression of both *ras-1* and *RAS2* takes a few days to present itself clearly. The reason for this could be that transformants of Δ*cit2* need to be streaked on synthetic glucose medium twice and then on synthetic glycerol medium, which results in very slow growth on the glycerol media of wild type strains in general. This step has to be taken because of the sensitivity of the Δ*cit2* mutant to complementation by trace amounts of carnitine. For example, glycerol media containing very small amounts of yeast extract or peptone complement the Δ*cit2* phenotype fully due to the large quantities of carnitine present

in these extracts. The suppression was also more prominent when transformants were spotted on amino acid selective glycerol plates.

4.3 *RAS2* and *ras-1* overexpression improves proliferation on glycerol

Ras is involved in a complex array of cellular processes and control mechanisms for metabolic regulation. Therefore, because of the far-reaching influence of Ras on metabolism, we determined if the suppression was specific to the Δ*cit2* mutant, or if it could be due to a general enhancement of proliferation on glycerol. The Ras genes were overexpressed in FY23 wild type and the growth monitored on glucose and glycerol (Figure 2). Indeed, overexpression of Ras in wild type cells improved the proliferation on minimal glycerol media. The wild type strains with overexpressed Ras reached a much higher optical density on glycerol at stationary phase compared to wild type. However, on glucose media, the opposite was observed. Strains with overexpressed Ras had a lower optical density at stationary phase compared to wild type. This would indicate that the high biomass yield reached at stationary phase is not a general effect present on all carbon sources.

The enhancement of proliferation by *RAS2* overexpression is in contrast to the well-known growth defect caused by the $RAS2^{val19}$ allele on glycerol. Indeed, FY23 wild type strains harbouring the $RAS2^{val19}$ allele showed a growth defect on glycerol media and reduced biomass yield at stationary phase (Figure 2). Interestingly, the highest optical density reached at stationary phase for strains growing on glycerol was observed in the FY23Δ*cit2* strains with *RAS2* overexpressed. In this case, carnitine was added to complement the Δ*cit2* mutant's growth on glycerol. However, adding carnitine to the FY23 wild type and the wild type overexpressing *RAS2*, did not change the optical density reached at stationary phase. Also, the Δ*cit2* mutant grew similar to wild type when carnitine was added. Therefore, the effect is not related to carnitine but to *RAS2* overexpression in the Δ*cit2* background. For the Δ*cit2* strain grown on glycerol medium containing carnitine (SCG + carnitine), a 2 fold increase in optical density at stationary phase was shown when *RAS2* was overexpressed. In the same conditions and in wild type, only a 1.3 fold increase in optical density was observed when *RAS2* was overexpressed. This would indicate regulatory effects between Ras2p and Cit2p. Indeed, it has been shown previously that *RAS2* regulates the retrograde response, which is the communication between the mitochondria and the nucleus in response to mitochondrial dysfunction or damage (Kirchman et al., 1999). The *CIT2* gene is known to be upregulated in response to mitochondrial dysfunction or damage (Liao et al., 1991). In our case, the absence of *CIT2* allows *RAS2* to enhance proliferation on glycerol significantly better than when *CIT2* is present.

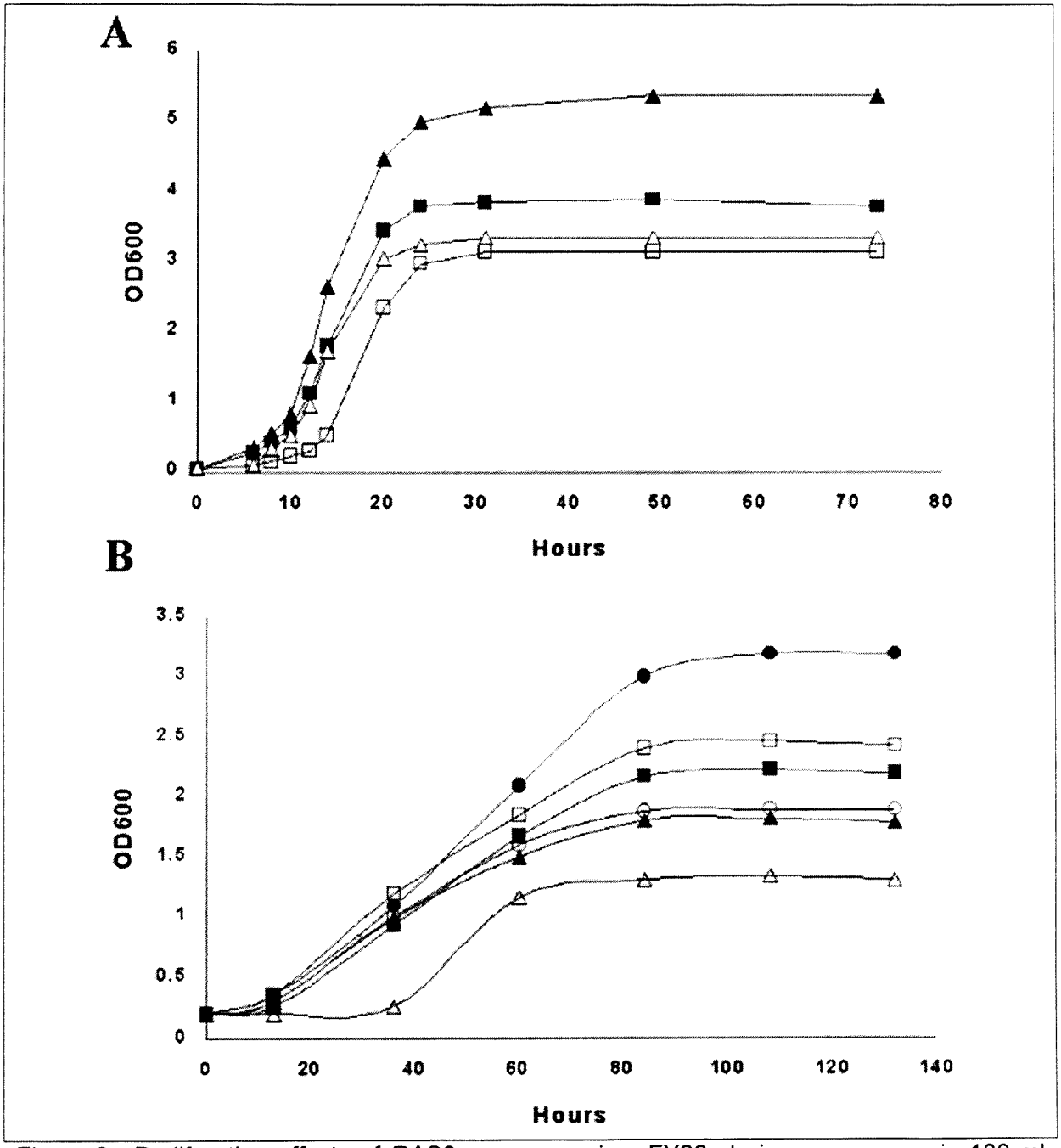


Figure 2. Proliferation effects of *RAS2* overexpression. FY23 strains were grown in 100 ml synthetic medium with 2% glucose (**A**) and synthetic medium with 3% glycerol (**B**). Precultures were grown in SCD media for 1 day and inoculated at OD_{600} of 0.05 for glucose growth curves and OD_{600} of 0.2 for glycerol growth curves. Symbols are as follows: Wild type strain (▲); Wild type strain with *RAS2* overexpressed (■); Wild type strain with *Nc-ras1* overexpressed (□); Wild type strain with $RAS2^{val19}$ (Δ); Δ*cit2* strain (O); Δ*cit2* strain with *RAS2* overexpressed (●). Strains were transformed with vector pHVXII or pHVXII-*RAS2* and growth curves were done in triplicate.

4.4 *RAS2* overexpression results in increased cAMP/PKA activity

The *RAS2*^{val19} allele translates a Ras protein that is locked in the GTP bound form and therefore constitutively activates the Ras/cAMP/PKA pathway through elevated intracellular cAMP concentrations (Toda et al., 1985; Nikawa et al., 1987). A large amount of Ras2p in the cell would not necessarily mean higher activity as would be the case for most enzymes. The level of activation of the cAMP/PKA pathway would depend on the relative amount of GTP bound Ras2p and not on the total amount of Ras2p present. In addition, various forms of regulation might influence this Ras2p-GTP/Ras2p-GDP ratio when these proteins are in abundance. Previous reports have indicated that overexpression of *RAS2* does not result in significant elevation of intracellular cAMP, thereby implying limited activation of the PKA pathway (Sun et al., 1994; Colombo et al., 1998).

In this work we show that overexpression of *RAS2* results in a decrease in glycogen content. However, the reduction in glycogen levels were less than in the case of *RAS2*^{val19} (Figure 3A). Also, *RAS2* overexpressed strains were sensitive to nutrient starvation (data not shown).

We also observed a flocculation phenotype in minimal glucose media when *RAS2* was overexpressed (Figure 3B). The Ras pathway is known to act on related phenotypes such as pseudohyphal differentiation and invasive growth (Mosch et al., 1999; Pan and Heitman et al., 1999). For instance, *FLO11*, which has been implicated in flocculation and invasive growth, is known to be upregulated when the Ras/cAMP/PKA pathway is activated (Pan and Heitman, 1999). We further showed that the Δ*tpk2* strain blocked the flocculation phenotype in *RAS2* overexpressed strains (data not shown). The blocking of invasive growth in Ras activated strains by the Δ*tpk2* deletion has previously been shown, confirming the correlation (Pan and Heitman, 1999). As expected, *RAS2*^{val19} also caused flocculation on synthetic glucose media. However, the flocculation was much more intense for strains with *RAS2*^{val19}.

To finally implicate *RAS2* overexpression in the activation of the cAMP/PKA pathway, we measured intracellular cAMP in S288c isogenic strains grown on glucose and glycerol media. Cells harvested in late stationary phase growing on glycerol showed a clear increase in intracellular cAMP levels when *RAS2* was overexpressed (Figure 3C). In addition, we also confirmed the hypothesis that a lower basal level of cAMP is required for growth on glycerol compared to glucose. The drop in cAMP in cells moving from exponential to stationary phase in glucose was also confirmed (Russel et al., 1993) Interestingly, cAMP was much higher in late stationary phase of glycerol grown cells compared to exponentially grown cells (Figure 4C).

Collectively, all these data would therefore indicate that overexpressing *RAS2* does result in the activation of the cAMP/PKA pathway.

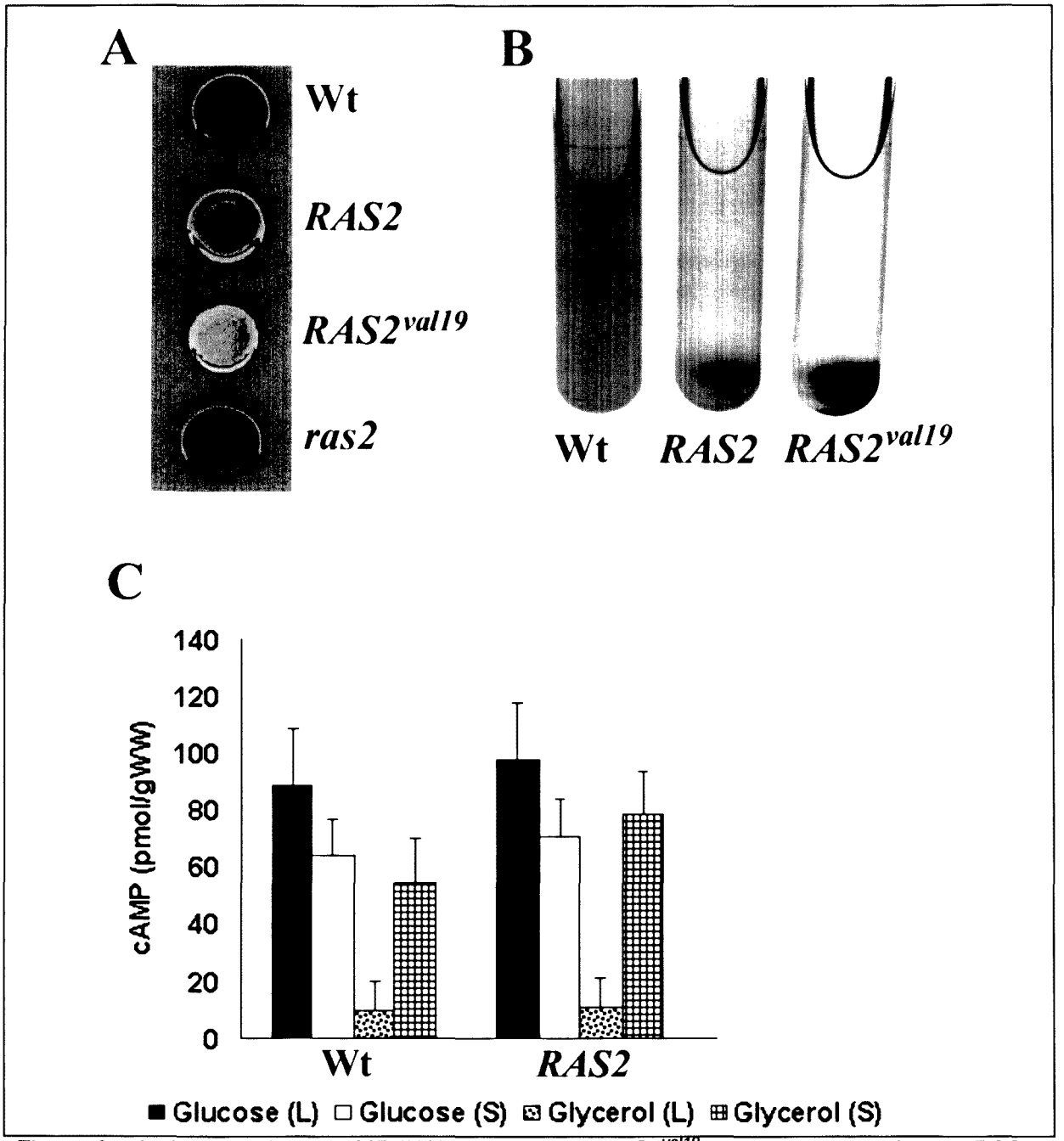


Figure 3. Activation of the cAMP/PKA pathway by *RAS2*^{val19} and overexpression of *RAS2*. (**A**) Iodene/iodide staining of transformants. Transformants were grown on SCD medium for 2 days and then spotted on SCD medium and grown for 5 days at 30°C. A solution of 0.2% iodine/0.4% potassium iodide was gently poured over the colonies and photographs taken 3 minutes later. The darker the colour, the more glycogen is present and lighter the colour, the less glycogen is present (**B**) Flocculation phenotypes. Strains were grown in SCD media for one day at 30°C on a rotating wheel and photographs taken. Optical densities did not vary more than 10%. (**C**). Intracellular cAMP measurements. Strains were grown on SCD and SCG media and harvested in log phase (L) or late stationary phases (S). Intracellular cAMP was measured as described in 'Materials and Methods'.

4.5 The *RAS2* proliferation effect is blocked in the Δ*tpk1* mutant

Ras2p can signal, independently of cAMP/PKA, through the MAPK cascade to promote filamentous growth and cell integrity (Lee et al., 1999; Mosch et al., 1999; Pan et al., 2000). Here we show that the proliferation effect of *RAS2* overexpression does act through the cAMP/PKA pathway. We overexpressed *RAS2* in BY4742 strains (Euroscarf), which carry deletions in the catalytic subunits encoded by *TPK1, TPK2* and *TPK3*. The growth of these strains was then monitored in glycerol media (Figure 4A).

In these conditions, the Δ*tpk3* strains showed a significant reduction in biomass yield at stationary phase compared to wild type. However, *RAS2* overexpression in Δ*tpk3* resulted in growth similar to wild type overexpressing *RAS2*. Therefore, *RAS2* also suppresses the growth defect on glycerol of the Δ*tpk3* strain. The Δ*tpk2* strain grew the same as the wild type strain. The Δ*tpk2* strain also grew identical to wild type when *RAS2* was overexpressed in both strains. The Δ*tpk1* strain had a higher biomass yield at stationary phase compared to the wild type strain. However, *RAS2* overexpression did not result in the high biomass yield observed for the wild type, Δ*tpk2* and Δ*tpk3* strains when *RAS2* was overexpressed. However, on synthetic glucose the characteristic reduction in biomass yield at stationary phase was observed for Δ*tpk1* when *RAS2* was overexpressed (as for all the other strains used) (Figure 4B). This would indicate that *RAS2* overexpression confers its proliferation effect of cells grown on glycerol through Tpk1p.

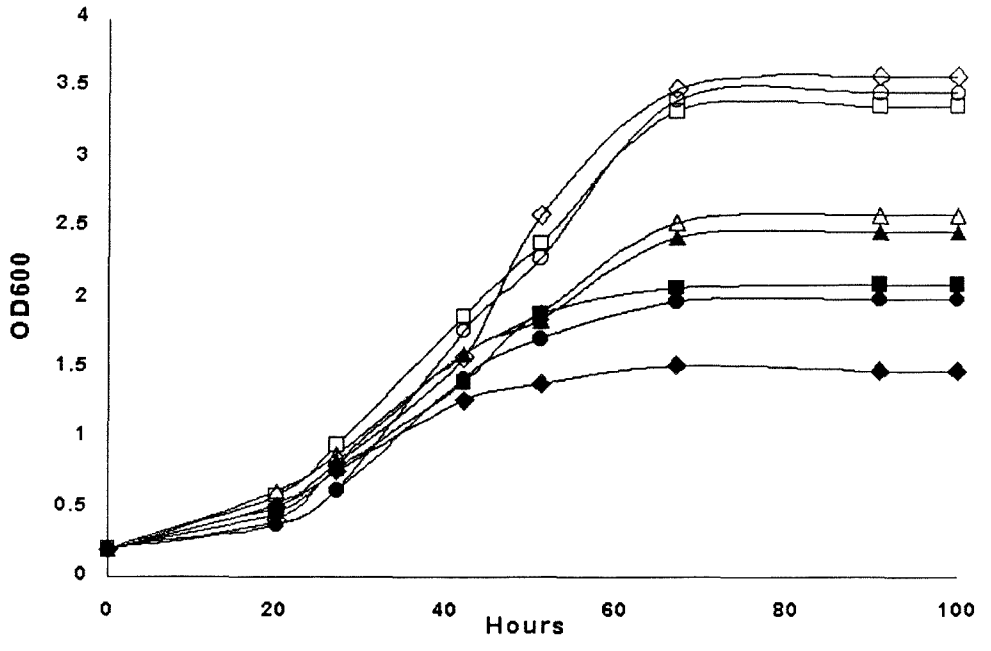
4.6 Overexpression of *RAS2* increases mitochondrial citrate synthase activity and intracellular citrate content

The Δ*cit2* mutant may be suppressed on non-fermentable carbon due to the leakage of mitochondrial citrate synthase and/or citrate to the cytosol. The *S. cerevisiae* genome encodes for three citrate synthases; the cytosolic Cit2p and the mitochondrial Cit1p and Cit3p (Kim et al., 1986, Rosenkranz et al., 1986; Jia et al., 1997).

In order to determine if Ras increases citrate synthase activity, enzyme assays were done on glucose and glycerol grown cells. On glycerol grown cells, *RAS2* overexpression increased citrate synthase activity and in the Δ*cit2* mutant citrate sythase activity almost doubled, indicating that the mitochondrial citrate synthase is upregulated (Figure 5). In contrast, the opposite effect was seen on glucose where citrate synthase activity decreased when *RAS2* was overexpressed. On glucose grown cells, no significant change in citrate synthase activity was monitored for the Δ*ras2* strain, but a significant drop in activity was seen when the strain was grown on glycerol medium. Interestingly, the $RAS2^{val19}$ strain showed increased citrate synthase activity in wild type cells grown on glycerol and decreased activity in wild type cells grown on glucose, similar to *RAS2* overexpression but in both cases more

pronounced. However, *RAS2*^{val19} could not suppress the Δ*cit2* mutant and caused a growth defect in wild type cells grown on glycerol (Figure 1 and Figure 2).

A



B

Strain	Wt	Wt *RAS2*	*tpk1*	*tpk1 RAS2*	*tpk2*	*tpk2 RAS2*	*tpk3*	*tpk3 RAS2*
OD_{600} Gluc	4.4	2.7	3.3	2.2	4.5	3.2	4.1	3.0

Figure 4. Proliferation effect of *RAS2* overexpression in Δ*tpk* strains. (**A**) Growth of BY4742 strains on SCD. Precultures were grown in SCD media for 1 day and inoculated at an OD_{600} of 0.2 to start the measurement of the growth curves. Symbols are as follows: Wild type strain (●); wild type strain with *RAS2* overexpressed (O); Δ*tpk1* strain (Δ); Δ*tpk1* strain with *RAS2* overexpressed (▲); Δ*tpk2* strain (■); Δ*tpk2* strain with *RAS2* overexpressed (□); Δ*tpk3* strain (◆) Δ*tpk3* strain with *RAS2* overexpressed (◇). Strains were transformed with vector pHVXII or pHVXII-*RAS2* and growth curves done at least in triplicate. (**B**) The average stationary phase OD_{600} reached for SCD grown BY4742 strains.

Intracellular citrate levels are an indication of respiratory activity in yeast. Citrate concentrations were therefore determined in *RAS2* overexpressed strains. The level of citrate in a wild type strain was 93 μg/gWW and in Δ*cit2* strains 46 μg/gWW. When *RAS2* was overexpressed citrate levels increased in wild type 2-fold to 201 μg/gWW and 2.5-fold more in the Δ*cit2* strain at 117 μg/gWW. Therefore, levels of citrate in the Δ*cit2* strains with overexpressed *RAS2* were even higher than in wild type strains.

From these data, it is clear that *RAS2* tightly regulates citrate synthase activity in the cell. This seems to be part of a regulatory circuit involved in upregulation of mitochondrial biogenesis and mitochondrial enzyme content. Therefore, possible leakage of mitochondrial citrate synthases and citrate from the mitochondria to the cytosol are probably part of general increase in respiratory and proliferation capacity of *RAS2* overexpressing cells grown on glycerol.

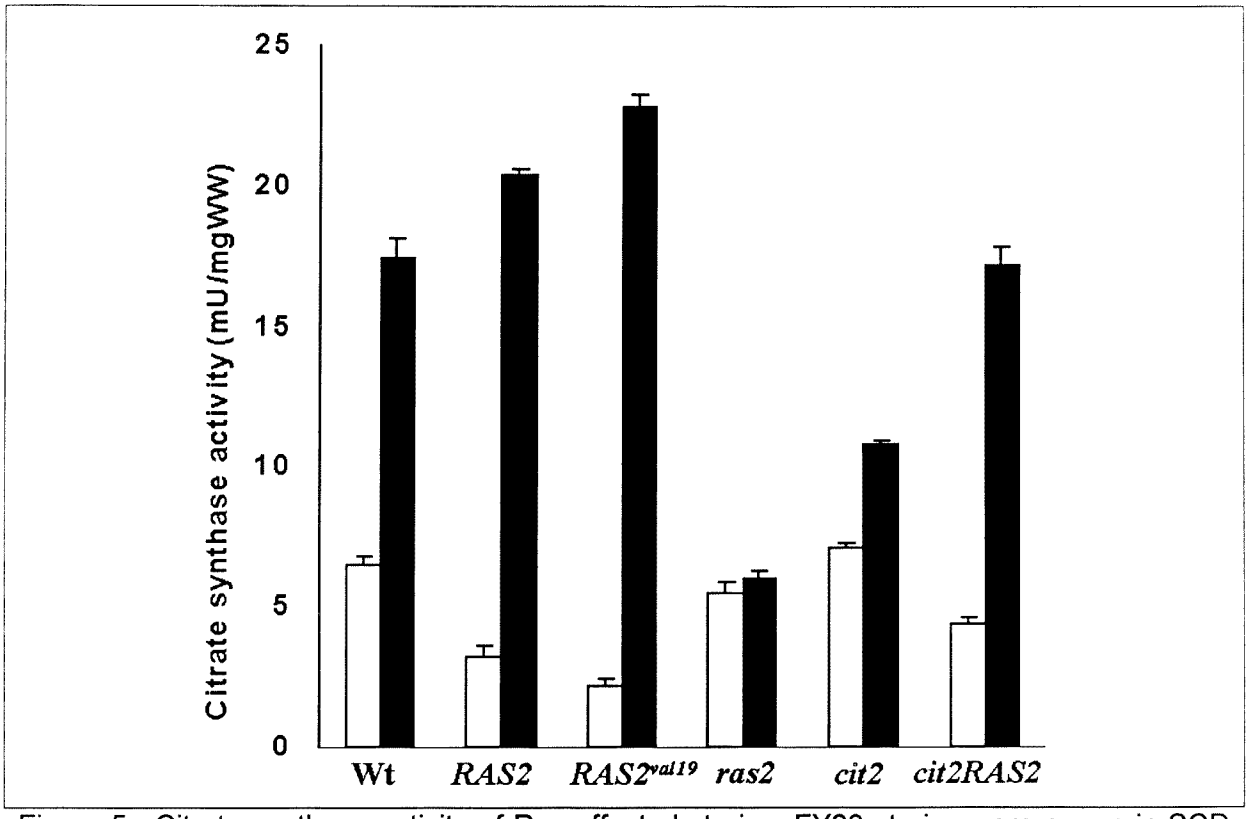


Figure 5. Citrate synthase activity of Ras affected strains. FY23 strains were grown in SCD (white bars) and SCG (black bars) media. For the Δ*cit2* stains, carnitine was added to complement growth on glycerol medium. The Δ*ras2* and *RAS2*^{val19} strains were inoculated at OD_{600} of 0.5 in SCG to allow growth to OD_{600} of 1. The SCD strains were grown for 24 hours and the SCG strains were harvested at OD_{600} of 1-1.5. Citrate synthase activity was measured as described in 'Materials and Methods'.

5. Discussion

In this work, we have identified the *N. crassa ras-1* gene as a suppressor of the *S. cerevisae* Δ*cit2* mutant when grown on glycerol medium. We subsequently showed that the native *RAS2* gene could also suppress the Δ*cit2* mutant when overexpressed. Further investigation indicated that *RAS2* overexpression in cells grown on glycerol: (i) enhances biomass yield of wild type; (ii) activates the cAMP/PKA pathway; and (iii) upregulates mitochondrial citrate synthase activity. We further showed that the increase in biomass yield in wild type cells grown on glycerol

and overexpressing *RAS2* is blocked in the Δ*tpk1* mutant. Recently, Tpk1p has been implicated in respiratory growth through its apparent regulation of iron content in the mitochondria (Robertson et al. 2000). In the same work, Tpk1p has also been shown to regulate Bat1p expression, which is involved in the regulation of stationary phase. Together, these data implicate the important role of Tpk1p in growth on non-fermentable carbon sources.

We also compared the effect of $RAS2^{val19}$ with that of *RAS2* overexpression and found that both result in: (i) a decrease in glycogen content; (ii) nutrient starvation sensitivity; (iii) the flocculation of cells grown on glucose; (iv) a decrease in biomass yield and citrate synthase activity for wild type cells grown on glucose medium; and (v) an increase in citrate synthase activity of wild type cells grown on glycerol. In support of the last point, it has previously been shown that cAMP addition increases *CIT1* expression and citrate synthase activity in cells grown on the non-fermentable carbon source lactate (Dejean et al., 2002). However, we show that all these phenotypes were more pronounced in the case of strains with the $RAS2^{val19}$ allele. Surprisingly, in reciprocal effects, *RAS2* overexpression enhanced proliferation and biomass yield of cells growing on glycerol compared to the decreased proliferation and biomass yield of cells with $RAS2^{val19}$ in these conditions.

The importance of the Ras/cAMP/PKA regulatory pathway in respiratory growth on non-fermentable carbon sources is well known. Most of the deletions in this pathway result in growth defects on glycerol (e.g. Δ*ras2*, Δ*bcy1*, Δ*ira1*Δ*ira2*), as does constitutive activation of the $RAS2^{val19}$ allele. In this work we also investigated the BY4742 strains with a deletion in the cAMP phosphodiesterase genes *PDE1* and *PDE2*. We found that deletion of *PDE2* (but not *PDE1*) displays a growth defect when grown on glycerol medium (data not shown). Therefore, over- and underactivation of the Ras/cAMP/PKA pathway results in growth defects on non-fermentable carbon sources. Indeed, recent data indicate that the Ras/cAMP/PKA pathway plays a much more important role on non-fermentable carbon sources than on fermentable carbon sources (Robertson et al., 2000). In addition, it has been shown that activation of the cAMP pathway increases mitochondrial enzyme content when the cells are grown on the non-fermentable carbon source lactate (Dejean et al., 2002).

In our work, and in contrast to the growth defects exhibited by the Δ*ras2* mutant, we show that *RAS2* overexpression enhances proliferation on glycerol. Other examples of these reciprocal effects are the effect of *RAS2* overexpression on life span extension vs. the effect of Δ*ras2* on life span curtailing (Sun et al., 1994). On the other hand, it has previously been shown that $RAS2^{val19}$ overexpression and Δ*bcy1* curtails life span (Sun et al., 1994; Pichova et al., 1997). These findings lead researchers in the field to propose that *RAS2* overexpression acts independently of the cAMP/PKA pathway (Sun et al., 1994). However, later, the same workers indicated that overexpression of *RAS2* can reverse the effect of chronic stress on life span strictly through the cAMP/PKA pathway (Shama et al., 1998). Our data on

proliferation of cells on glycerol as a sole carbon source correlate with these observations. In our case, we show that *RAS2* overexpression enhances proliferation of cells growing on glycerol in contrast to the growth defect of Δ*ras2* in these conditions. We show that the *RAS2* overexpression proliferation effect is mediated by the cAMP/PKA pathway. Recent data indicate that $RAS2^{val19}$ can act independently of the cAMP/PKA pathway to generate reactive oxygen species and lock respiration in a non-phosphorylating state (Hlavata et al., 2003). These cAMP/PKA independent effects of $RAS2^{val19}$ may contribute to the growth defect of cells grown on glycerol. This is supported by the fact that, on non-fermentable carbon sources, both $RAS2^{val19}$ and *RAS2* overexpression result in an increase in mitochondrial enzyme content (including citrate synthase activity and ATPase activity) (Dejean et al., 2002; Mabuchi et al., 2000). However, we show that *RAS2* overexpression and not $RAS2^{val19}$, improves proliferation of cells grown on glycerol. The more pronounced activation of the cAMP/PKA pathway by $RAS2^{val19}$ may also be responsible for the adverse effect regarding growth on glycerol. This is supported by the phenotype of the Δ*bcy1* mutant, showing that constitutively active PKAs result in a growth defect on glycerol. Therefore, *RAS2* overexpression might activate the cAMP/PKA at the right level in order to alter the mitochondrial machinery in a positive way to promote growth and proliferation on glycerol.

In support of our data, it has been shown that the ATPase mutant (*atp1-2*), which has a growth defect on glycerol, could be suppressed by overexpression of *RAS2* on a multi-copy plasmid under its native promotor (Mabuchi et al., 2000). It was also shown that *RAS2* overexpression increases ATPase activity and deletion of *RAS2* decreases ATPase activity of cells grown on glycerol medium. This correlates with our data regarding citrate synthase activity. In our case, ATPase activity should also increase when *RAS2* is overexpressed in cells grown on glycerol medium. The high biomass yield achieved in these conditions would support this notion, as more ATP would be required for this process. In conclusion, it is clear that *RAS2* overexpression in *S. cerevisiae* alters the mitochondrial metabolism and regulation in a way that supports growth on glycerol.

We propose that the suppression of the Δ*cit2* mutant on glycerol by *RAS2* overexpression is a combination of indirect effects related to mitochondrial capacity. The overexpression of *RAS2* in cells grown on glycerol results in: (i) enhanced proliferation of wild type strain and Δ*cit2* strain; and ii) increase in citrate synthase activity in wild type strain and Δ*cit2* strain. However, in both cases the effects were much more pronounced in the Δ*cit2* strain. It would therefore seem that there is some type of communication between the glyoxylate cycle and Ras2p with regards to mitochondrial regulation, which allows the cell to upregulate its mitochondrial machinery, even better than is the case for wild type. This could result in citrate synthase/citrate leakage to the cytosol, alteration in metabolic pools of the

mitochondria, and superior respiratory growth machinery, which together, would suppress the growth defect of the Δ*cit2* strain when grown on glycerol medium.

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CHAPTER 7

Concluding remarks and future perspectives

1. Concluding remarks and future perspectives

At the start of this research programme, components of the carnitine shuttle had to be identified. Work by van Roermund et al. (1995) provided important insights and allowed the design of a specific genetic screen for the isolation of genes involved in carnitine-dependent metabolism. A key contributor to the selection strategy and work described in Chapters 3-6 was the use of the Δ*cit2* mutant, carrying a deletion of the glyoxylate cycle specific citrate synthases gene. The deletion of this gene caused the strain to be completely dependent on exogenous carnitine for growth on non-fermentable carbon sources, an ideal genetic background to isolate carnitine shuttle mutants. Mutants in three of the four genes known to be involved in carnitine metabolism were identified. This included a novel gene, *YER024w*, that we named *YAT2*. Assays confirmed that *YAT2* encodes a carnitine acetyltransferase (CAT) and that it contributes greatly to CAT activity in cytosolic enriched fractions (Swiegers et al., 2001). We speculated that *YAT2* encodes a cytosolic CAT, similar to the recently identified cytosolic CAT of *Aspergillus nidulans*, FacC (Stemple, 1998). Kroppenstedt and co-workers later showed through green fluorescence protein (GFP) fusions that Yat2p is indeed located in the cytosol (Kroppenstedt et al., unpublished). It is not clear why there is a need for a cytosolic CAT in yeast and fungi but it could be that it is required for metabolism of carbon sources such as ethanol and acetate, which results in the production of acetyl-CoA in the cytosol (Schmalix and Bandlow, 1993; Stemple et al., 1998).

It was shown that all three CATs are essential in the carnitine-dependent Δ*cit2* strain. Therefore, deletion of *CAT2*, *YAT1* or *YAT2* renders the carnitine shuttle non-functional. Kroppensted and co-workers also showed that over-expression of any of the three CATs does not result in cross-complementation, indicating that each CAT performs a very specific metabolic function (Kroppenstedt et al., unpublished). In future, it would be important to uncover the specific role of each CAT in *S. cerevisiae*. It may be that these CATs can be involved in other functions besides the role in transferring acetyl groups to the mitochondria. In particular, the role CAT could play in the regulation of acetyl-CoA/ CoA pools, as was shown for human carnitine acyltransferases (Bremer, 1983). Or, in a less defined manner, the effect on osmotic stress resistance, similar to what was shown for the effect of carnitine in protecting microorganisms (Verheul et al. 1997; Verheul et al. 1998; Angelidis et al. 2002; Mendum and Smith, 2002; Wemekamp-Kamphuis et al. 2002; Angelidis et al. 2003). Indeed, Font-Sala and co-workers showed that carnitine results in stress resistance and osmotolerance in *S. cerevisiae* (Font-Sala et al., unpublished). In support of this, it has recently been shown that the carnitine plasmamembrane transporter, Agp2p, is induced upon osmotic shock (Lee et al., 2002). Although the metabolic reasons for these stress resistance effects are not clear, transcriptional data derived in these

conditions may supply some clues. Indeed, it was recently shown that the *CAT2* gene is strongly induced during heat shock (Gasch et al. 2000).

The CATs have also been implicated in various other cellular processes. In work done in the laboratory of SG Oliver (University of Manchester) it was shown that *YAT2* was highly expressed during stationary phase (personal communication). Also, Lorenz and Fink (2001) showed that *YAT2* is highly induced during phagocytosis, thereby implicating that carnitine acetyltransferases might be involved in virulence of the pathogen *Candidia albicans*.

Although it is clear that carnitine plays an important role in the metabolism of *S. cerevisiae*, it is surprising that it was only recently discovered that this organism does not biosynthesise carnitine (van Roermund et al., 1999; Swiegers et al., 2001; Swiegers et al., 2002). However, it does efficiently take up carnitine when grown on non-fermentable carbon sources.

The absence of carnitine biosynthesis in *S. cerevisiae* has important metabolic consequences when cells are grown on synthetic media. However, trace amounts of carnitine are required in order for the carnitine shuttle to function properly. The components of rich media (peptone and yeast extract) contain sufficient amounts of carnitine, therefore explaining the lack of phenotype previously observed for these strains on this media (Kim et al., 1986; Kispal et al., 1988).

It is not clear why *S. cerevisiae* does not biosynthesise carnitine. It may be that in conditions where this yeast thrives, high glucose and anaerobiosis, carnitine is not essential for growth. In industrial conditions, such as during wine and beer fermentation, carnitine is present in sufficient quantities (Swiegers et al., unpublished). However, we showed that *Yarrowia lipolytica*, *Rhodotorula graminis* and *Candida curvata* do biosynthesise carnitine. This could be related to efficient metabolism of fatty acids, requiring carnitine to facilitate the oxidation process.

The genetic introduction of the carnitine biosynthesis pathway in *S. cerevisiae* could be of great scientific and commercial benefit. In general, carnitine biosynthesising yeast would be beneficial for consumers of bread, wine and beer. The clear benefit of carnitine to human health was discussed in Chapter 2.

Chapters 4 and 5 describe the first molecular work done on carnitine biosynthesis related to eukaryotic microorganisms. It was interesting to find that the filamentous fungi *Neurospora crassa* has an out of the ordinary protein sequence for the two biosynthesis genes encoding BBH and TMLH, which contained repeat regions that did not influence the activity of the enzymes. Recently, the complete genome of this organism was sequenced, making this a well-suited organism to genetically study carnitine biosynthesis (Gallagan et al., 2003). It will be a challenging task to identify the other two biosynthesis genes from *N. crassa* because there is not enough molecular information available. The use of *N. crassa* biosynthesis mutants might overcome these obstacles.

We also found that the Δ*cit2* mutant could be suppressed by Ras proteins when grown on synthetic medium with glycerol as sole carbon source. The role of Ras in signalling during growth on fermentable carbon sources such as glucose has been intensively studied (Broach, 1991; Thevelein, 1994). However, much less is known about the role of Ras in the regulation of metabolism during growth on non-fermentable carbon sources, except for the well-known growth defect caused by the Δ*ras2* mutant and by the *RAS2*^{val19} allele on non-fermentable carbon sources (Tatchell et al., 1985). In fact, before the work described in Chapter 6 commenced, only one publication was available which linked the suppression of mutants grown on glycerol and Ras proteins (Mabuchi et al. 2000). In this work it was shown that *RAS2* suppresses the mitochondrial *atp1-2* mutation of cells grown on glycerol medium. In our work, the deleted Δ*cit2* mutant could be completely suppressed by both *RAS2* and *N. crassa ras-1*, indicating the important regulatory role of the Ras protein in growth of cells on on non-fermentable carbon sources.

We related the phenotypes of the overexpressed Ras directly to the cAMP/PKA pathway. We also show that Ras overexpression enhances the proliferation of *S. cerevisiae* on glycerol. In support of our findings, it has recently been proposed that Ras and exogenous cAMP increases mitochondrial enzyme content on the non-fermentable carbon source, lactate (Dejean et al. 2002). We show that *RAS2* overexpression increases citrate synthase activity and intracellular citrate levels in *S. cerevisiae*. We propose that a general upregulation of respiratory capacity coupled with the leakage of citrate synthase/citrate from the mitochondria suppresses the Δ*cit2* mutant when grown on glycerol medium.

These results indicate that there is still a lot to be learned about the role of Ras in regards to the regulation of growth on non-fermentable carbon sources. To date, this area of research has not received much attention. It is clear that Ras is a key element in regulation of metabolism on non-fermentable carbon sources. In future, it may become an upstream target for metabolic manipulation for desired industrial purposes, including controling ethanol levels and biomass yields.

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APPENDIX

The determination of carnitine acetyltransferase activity in *Saccharomyces cerevisiae* by HPLC-electrospray mass spectrometry

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The determination of carnitine acetyltransferase activity in *Saccharomyces cerevisiae* by HPLC-electrospray mass spectrometry

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1. Abstract

Carnitine acetyltransferases (CATs) catalyse the transfer of activated acetyl groups between the acetylated and free forms of coenzyme A and carnitine. This reaction is part of the carnitine shuttle, a metabolic pathway that is required for the exchange of activated acetyl groups between cellular compartments. Indeed, only acetyl-carnitine, and not acetyl-CoA, can be transported across intracellular membranes. Three CAT enzymes, encoded by the genes *CAT2*, *YAT1* and *YAT2*, have been identified in the model organism *Saccharomyces cerevisiae*. While genetic evidence suggests that each CAT plays a unique role in cellular metabolism, our current understanding of the metabolic role of CATs does not explain why three independent enzymes are required. To investigate the specific role of each CAT, an accurate method for the determination of CAT activity is required. Methods employing spectrophotometric measurements are unreliable, particularly when crude homogenate is used. A radiolabeled forward exchange assay can be used to eliminate this problem, but safety concerns make this method undesirable. Here we have developed an HPLC tandem electrospray mass spectrometric method for the measurement of CAT activity in crude cellular homogenate. In this method, the conversion of carnitine to acetylcarnitine is measured quantitatively over a period of time by monitoring the precursor ion containing the m/z = 85 fragment, which is unique to the acetylcarnitine molecule. Crude extract from a mutant strain with all three CAT-encoding genes deleted showed no CAT activity when this method was employed, indicating that there are only three CATs present in *S. cerevisiae*.

2. Introduction

Carnitine acetyltransferases (EC 2.3.1.7) are present in eukaryotic organisms and have been described in yeast, fungi, plants and mammals (Burgess and Thomas,

1986; Masterson and Wood, 2000; Jernejc and Legisa, 1996; Kozulic et al., 1987; Stemple et al., 1998; Bieber, 1988). They catalyse the transfer of activated acetyl groups from acetyl-CoA to L-carnitine or from acetylcarnitine to CoA-SH. This reaction is required because organellar membranes are impermeable to acetyl-CoA (Van Roermund et al., 1995), while a carnitine-acetylcarnitine antiport system can ensure the transfer of the activated acetyl group without loss of energy (Palmieri et al., 1999; Van Roermund et al., 1999).

In the model organism *Saccharomyces cerevisiae*, three genes code for carnitine acetyltransferases, *CAT2*, *YAT1* and *YAT2* (Stemple et al., 1998; Bieber, 1988; Schmalix and Bandlow, 1993; Kispal et al., 1991; Swiegers et al., 2001). The three enzymes have unique metabolic functions, since all three are essential in some genetic backgrounds and no cross-complementation can be observed (Swiegers et al., 2001; Van Roermund et al., 1995). In order to obtain a better understanding of the metabolic role of CAT enzymes in yeast, the activity of these enzymes has to be measured collectively and individually in cells grown in various conditions and an accurate method to measure CAT activity in crude cellular extracts is required. Existing methods to measure CAT activity depend on spectrophotometric measurements of either the formation or disappearance of acetyl-CoA derivatives at 232 nm, or the indirect measurement of the release of CoA-SH with sulfhydryl reagents. These methods are inaccurate when crude cellular homogenates are used, due to the high background absorbance (Colowick and Kaplan, 1986). The problem associated with the spectrophotometric assays has lead researchers to centrifuge crude cellular homogenate, using the clear, cell-free supernatant for the assay (Schmalix and Bandlow, 1993; Kispal et al., 1991; Swiegers et al., 2001). This practice, however, is not optimal, as some enzyme inevitably will be lost. A possible alternative is the use of a radioisotope exchange forward assay, but the need for specialised reagents, working space and equipment, as well as safety concerns when working with radioactive reagents, makes this method undesirable for routine assays (Demaugre et al., 1988; McGarry et al., 1983; Stakkestad and Bremer, 1982).

Previously, electrospray mass spectrometry was used by Nolte et al. (1998) to measure the activity of carnitine palmitoyltransferase I in leucocytes. The method was shown to be accurate and the specific activity of the enzymes compared well when other methods of determination were used. Here we present a method based on tandem mass spectrometry, in which the precursor ions containing the *m/z* = 85 fragment, which is unique to acetylcarnitine molecules, can be detected. Separation of the components in the reaction mixture by reverse phase high performance liquid chromatography (HPLC) was combined with tandem electrospray mass spectrometry (MSMS). The detection mode employed was multiple reaction monitoring, in which the first analyser of a triple quadropole mass spectrometer was set to pass the precursor ion (acetylcarnitine) into the fragmentation cell, where collision-induced dissociation created the *m/z* = 85 fragment, which was detected by the second

analyser. The combination of both HPLC and MS/MS provides a highly selective and specific method for the detection and quantification of acetylcarnitine in the presence of high concentrations of L-carnitine. No extraction steps are required in this assay, contributing to the simplicity and accuracy of the method.

3. Materials and methods

3.1 Yeast strains and plasmids

All strains used in this study derive from FY23 and are listed together with the plasmids in Table 1. For the cloning and disruption of the genes, standard DNA techniques were used (Sambrock et al., 1989).

Table 1. Strains and plasmids used in this study

Strains and plasmids	Genotype	Source or reference
Strains:		
FY23	*MATa leu2 trp1 ura3*	Winston et al. 1995
FY23Δ*yat2*	*MATa trp1 ura3 yat2::LEU2*	Swiegers et al. 2001
FY23Δyat2Δ*cat2*Δ*yat1*	*MATa yat2::LEU2 cat2::URA3 yat1::TRP1*	This study
Plasmids:		
YDp-W	*TRP1*	Berben et al. 1991
Ydp-U	URA3	Berben et al. 1991
PGEM-T-easy		Promega
PGEM-T-easy-*CAT2*		This study
pΔ*cat2*	Δ*cat2::URA3*	This study
pΔ*yat1*	Δ*yat1::LEU2*	Swiegers et al. 2001
pΔ*yat1*	Δ*yat1::TRP1*	This study

3.2 Cloning and disruption of genes

To create a yeast strain without any known CAT-encoding genes, the *CAT2* and *YAT1* genes were deleted from strain FY23Δ*yat2* (Swiegers et al., 2001). A 595 bp *Bam*HI/*Bgl*II fragment internal to the *CAT2* gene of the plasmid PGEM-T-easy-*CAT2* was replaced by a 1.1 kb *URA3* gene from plasmid YDp-U. The resulting construct, pΔ*cat2*::*URA3*, was used to isolate a 2.9 kb disruption cassette containing the *URA3* gene plus *CAT2* flanking regions, which was then transformed into FY23Δ*yat2*. Transformants were isolated on selective media and the disruption was verified using the primers CAT2F1 and CATR1. The Δ*yat1* disruption construct was created using the plasmid pΔ*yat1*::*LEU2* and replacing the *LEU2* gene by removing it as a 1.6 kb *Bam*HI fragment and replacing it with a 0.8 kb *TRP1* fragment from plasmid YDp-W. The resulting disruption construct, pΔ*yat1*::*TRP1*, was used to isolate a 1.9 kb

fragment containing the *TRP1* gene and *YAT1* flanking regions, which was transformed into strain FY23Δ*yat2*::*LEU2* Δ*cat2*::*URA3*. The disruptions were verified using primers YAT1F1 and YAT1R1.

3.3 Cultivation and homogenisation of yeast

Strain FY23 wild-type was grown in YND medium [0.67% (w/v) YNB without amino acids (Difco) and 2% (w/v) glucose] to an optical density (OD_{600}) of 3.0, corresponding to ca. 1.2 x 10^8 cells/ml. From this preculture, YNE medium [0.67% (w/v) YNB without amino acids and 2% (v/v) ethanol] was inoculated to an initial OD_{600} of 0.15 and the cells were grown to an OD_{600} of 1.0. A 40 ml sample of this culture was centrifuged at 5000 rpm for 5 min and the cells were washed with 40 ml of water and again centrifuged at 5000 rpm for 5 min. After resuspension in 1 ml of 15 mM KCl and 5 mM TRIS buffer (pH 7.4), the cells were transferred into 1.5 ml microcentrifuge tubes and centrifuged for 3 min at 5000 rpm. The excess water was removed and the pellet was resuspended in 200 µl ice-cold 0.05% (w/v) Triton X-100. Glass beads (0.16 g) were added to the suspension and the cells were vortexed for 10 min at 8°C. 100 µl of the crude extract were transferred to new microcentrifuge tubes and buffered with ice-cold 900 µl 15 mM KCl and 5 mM TRIS buffer (pH 7.4). The crude extract was kept on ice and used for the enzyme assay the same day. The total protein content was measured using the bicinchonic acid (BCA) kit.

3.4 Carnitine acetyltransferase assay

The assay mixture consisted of a buffer containing 13.5 mM KCl and 4.5 mM TRIS (pH 7.4), complemented with 0.049 mM acetyl-CoA and 2.47 mM L–carnitine. After a preincubation of 10 min at 30°C, the reaction was initiated by the addition of 10 µl of cell extract with a concentration of 1.3 mg/l total proteins. To measure the formation of acetylcarnitine, samples were taken at 10 min intervals by removing 100 µl aliquots from the sample mixture. These were added to 900 µl acetonitrile, thereby stopping the reaction. The samples were kept on ice and then deproteinised by centrifugation at 12000 rpm for 5 minutes. The supernatants were transferred to new tubes and analysed by HPLC/MS/MS.

3.5 Mass spectrometry

Mass spectrometry was performed on a Micromass (Manchester, UK) Quattro triple quadropole mass spectrometer fitted with an electrospray ionisation source. Solvent A (acetonitrile/water/formic acid: 30/70/0.05(v/v/v)) was used as carrier solvent and was supplied to the ionisation source by an LKB/Pharmacia (Sweden) pump. For

direct injection of the acetyl carnitine standard, the flow rate was 20 µl/minute and 5 µl of the standard was injected through a Rheodyne injection valve. The molecular ion ($[M+H]^+$) of acetylcarnitine was observed using a capillary voltage of 3.5 kV, a source temperature of 80°C and a cone voltage setting of 20 V. To obtain the fragment pattern of acetylcarnitine, the molecular ion was dissociated in the fragmentation cell by collision-induced dissociation at an argon pressure of 2.8 $X10^{-3}$ mbar, applying a collision energy of 20 eV. The resultant fragments were scanned in the second analyser.

Quantification of acetylcarnitine in the incubation samples was accomplished by HPLC/MS/MS. A Phenomenex (Torrance, USA) Luna C8 150X2.00 mm (3µ) column was used for separation, with solvent A as mobile phase at a flow rate of 100 µl/minute delivered by the above mentioned pump. Ten microlitres of sample were injected by a Waters 747 (UK) autosampler. The eluent from the column was directed into the electrospray ionisation source of the mass spectrometer. The capillary voltage, cone voltage, argon pressure and collision energy used were as mentioned above. Detection was by multiple reaction monitoring, using the molecular ion of acetylcarnitine as precursor ion and the fragment at *m/z* = 85 as product ion. Chromatographic peaks representing acetylcarnitine were integrated and the concentration in the incubation samples was calculated from a dilution range of known concentrations of standard acetylcarnitine, prepared in the same buffer as used for the enzyme assay and diluted to a final concentration of 90/10 (v/v): acetonitrile/15 mMTris.HCl. The calculations were performed automatically by the Quantify program of the MassLynx program and expressed as ng/ml.

4. Results

The electrospray mass spectrum of acetylcarnitine is depicted in Figure 1A. The molecular ion ($[M+H]^+$) at *m/z* = 204 is observed as the base peak in the spectrum. Fragmentation of the molecular ion resulted in the fragmentation spectrum shown in Figure 1B. Using the same conditions, the molecular ion of carnitine was detected at *m/z* = 164 and fragmentation produced an ion spectrum in which the most abundant fragment ion was observed at *m/z* = 43 (not shown).

For the quantification of acetylcarnitine, the molecular ion at *m/z* = 204 and the fragment ion at *m/z* = 85 were used as precursor- and product ions in the multiple reaction monitoring mode of detection. Carnitine was detected by using the precursor- and product ions at *m/z* = 164 and 43 respectively, in the multiple reaction monitoring mode. The components of the assay mixture were separated by HPLC before detection, and Figure 2 indicates that there is no cross-over contamination of the acetylcarnitine due to the presence of carnitine in the reaction mixture.

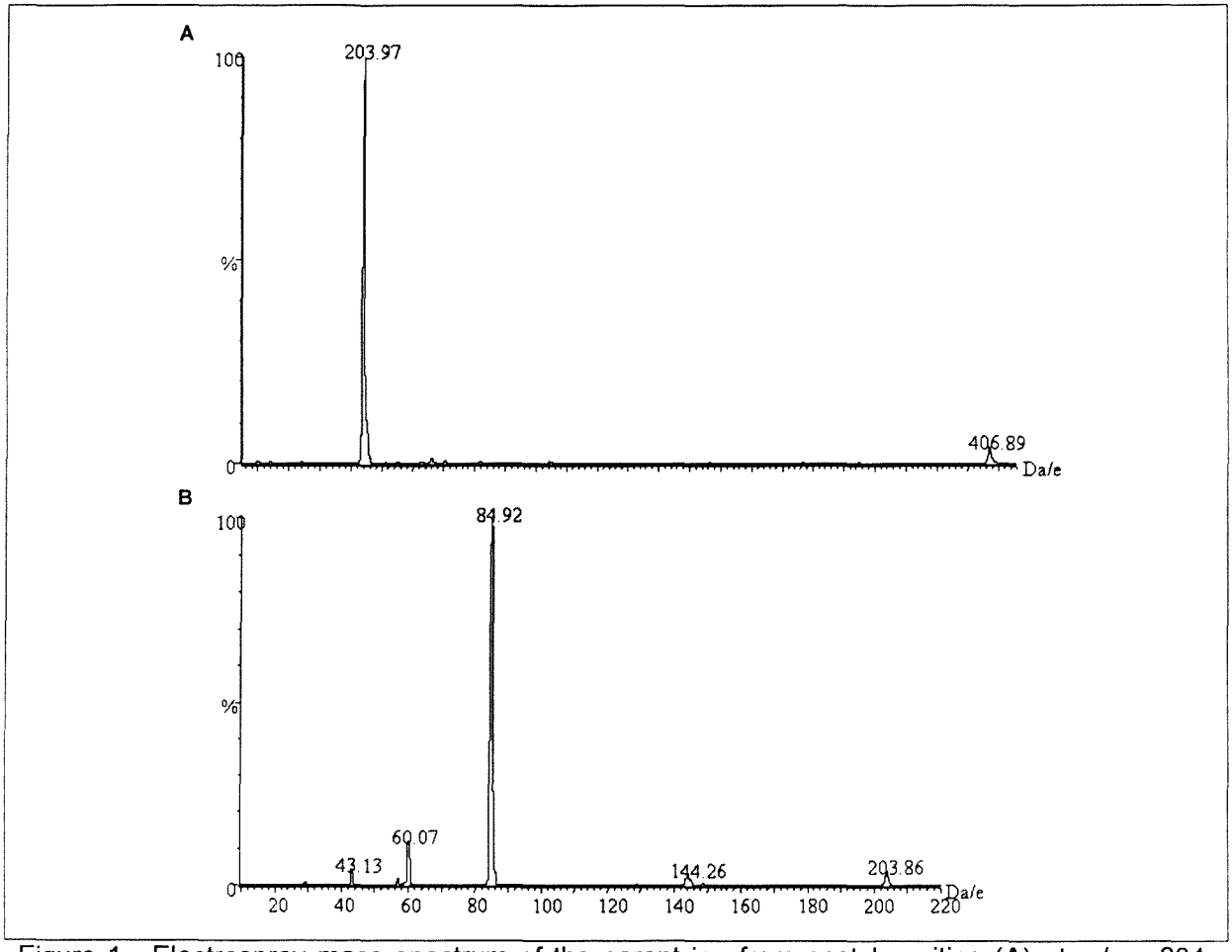

Figure 1. Electrospray mass spectrum of the parent ion from acetylcarnitine (**A**) at m/z = 204. The fragmentation resulted in the fragmentation ion at m/z = 84 (**B**), which was used for detection.

The concentration of acetylcarnitine in each incubation sample was calculated from a calibration curve established by using known concentrations of acetylcarnitine analysed under the same conditions as those used for the assay samples. The standard curve, with a coefficient of determination of 0.9990, is depicted in Figure 3.

Figure 4 represents the results obtained when CAT activity was measured in a wild-type yeast strain grown on ethanol. A linear increase in the conversion of acetyl-CoA to acetylcarnitine is clearly observed. The absence of acetylcarnitine at time zero is indicated clearly. The zero value was not included in the determination of activity, however, as some acetylcarnitine may have been transferred from the yeast crude extract. The specific CAT activity measured in these conditions was 7 pmol of acetylcarnitine formed per min and ng of protein. The result was highly reproducible in six independently conducted experiments (Figure 4).

In a second set of experiments, the effect of various protein concentrations on the formation of acetylcarnitine was measured. Here too, a near linear increase in product formation for increasing protein concentration over a fixed period of time was

observed. The reaction was stopped either after 20 min (Figure 5A) or after 40 min (Figure 5B).

As a control, the mutant strain with the three deleted *CAT* genes showed no activity, even when grown on other non-fermentable carbon sources, including oleic acid and acetate (data not shown). These data confirm that *S. cerevisiae* has only three CAT-encoding genes. In a second control, a reaction mixture containing distilled water instead of carnitine also showed no activity.

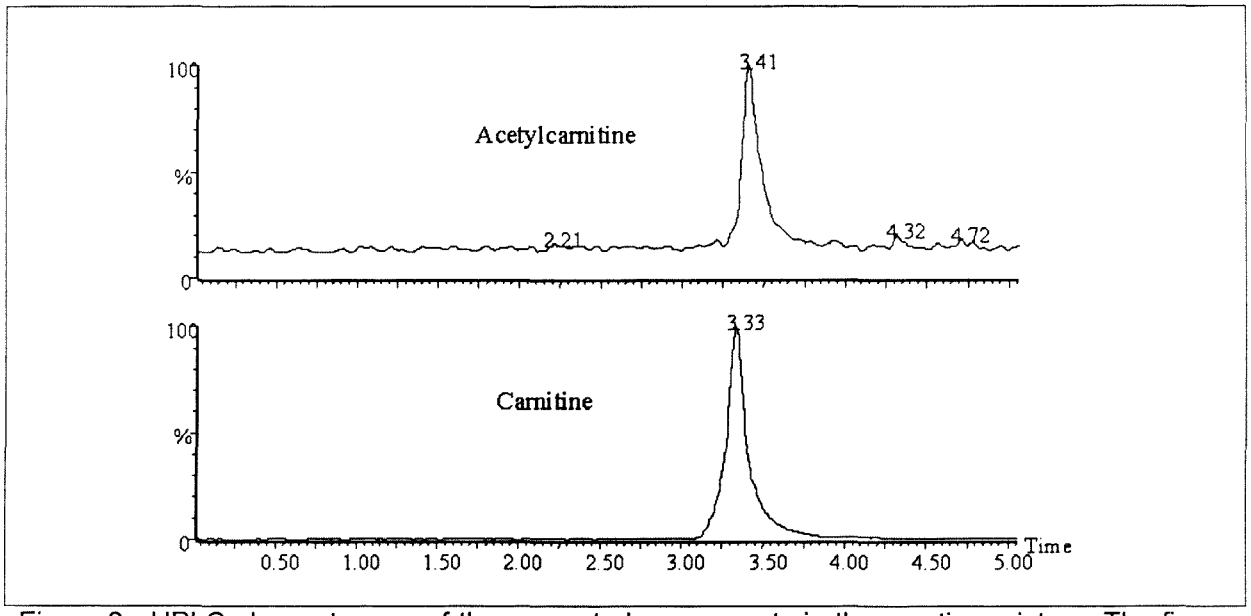

Figure 2. HPLC chromatogram of the separated components in the reaction mixture. The figure clearly indicates that there is no crossing over.

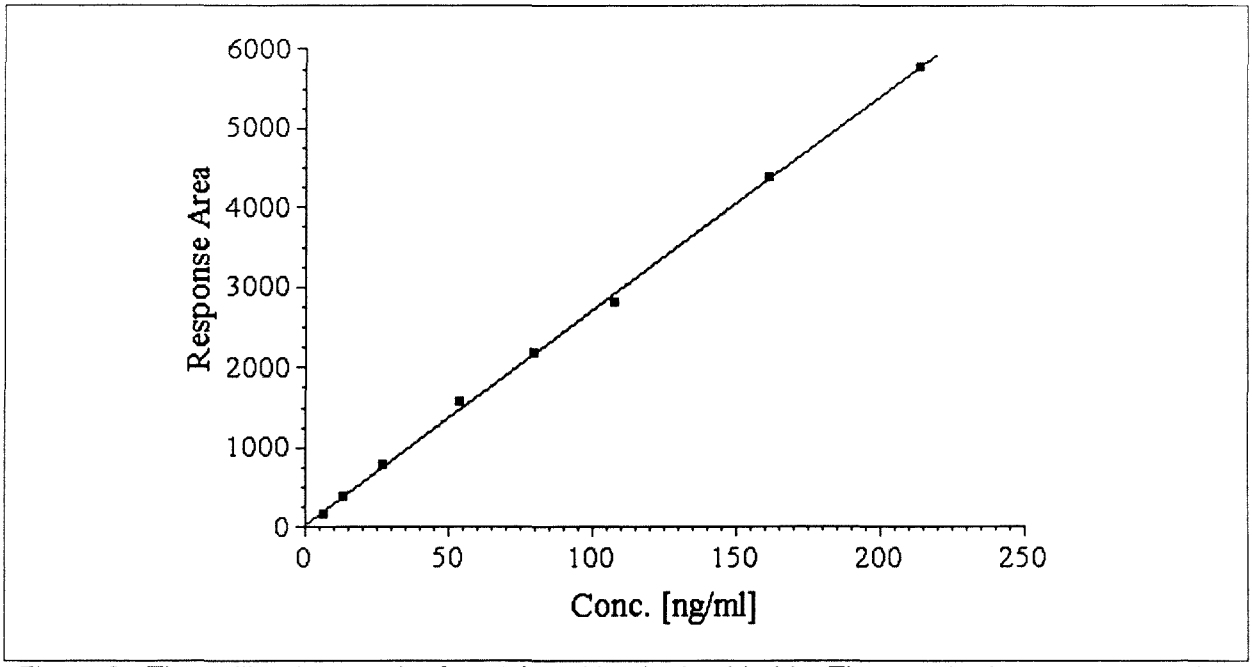

Figure 3. The calibration graph of acetylcarnitine-hydrochloride. The standard curve was used to determinate the concentration of acetylcarnitine formed during the enzyme assay.

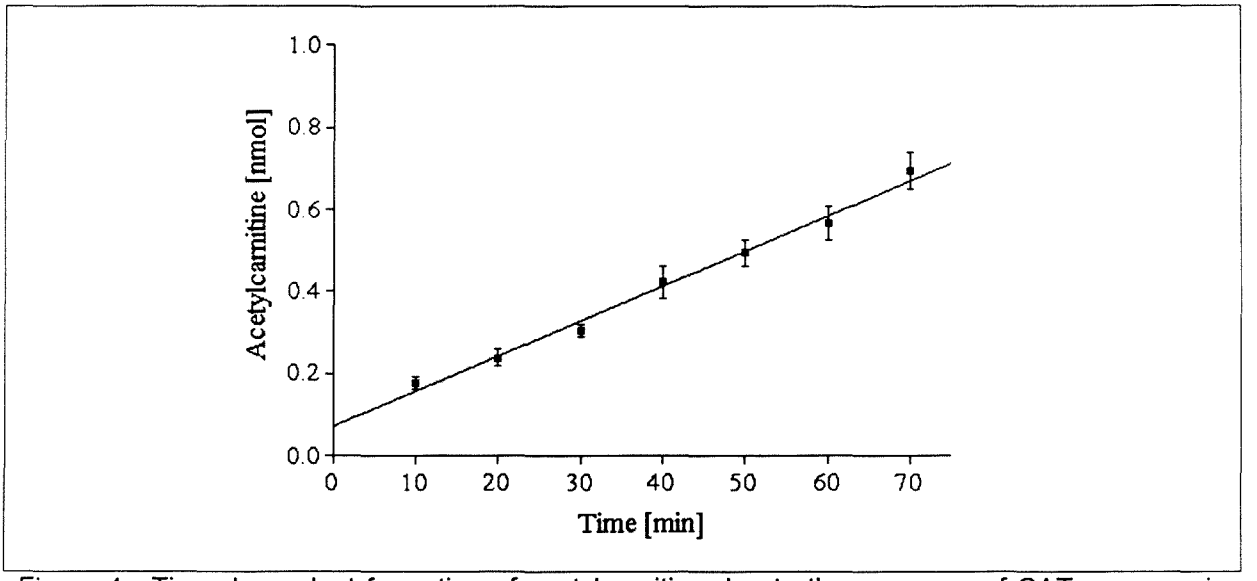

Figure 4. Time-dependent formation of acetylcarnitine due to the presence of CAT enzymes in the yeast crude extract. The reaction was performed as described under materials and methods. The graph presents the average of six repetitions.

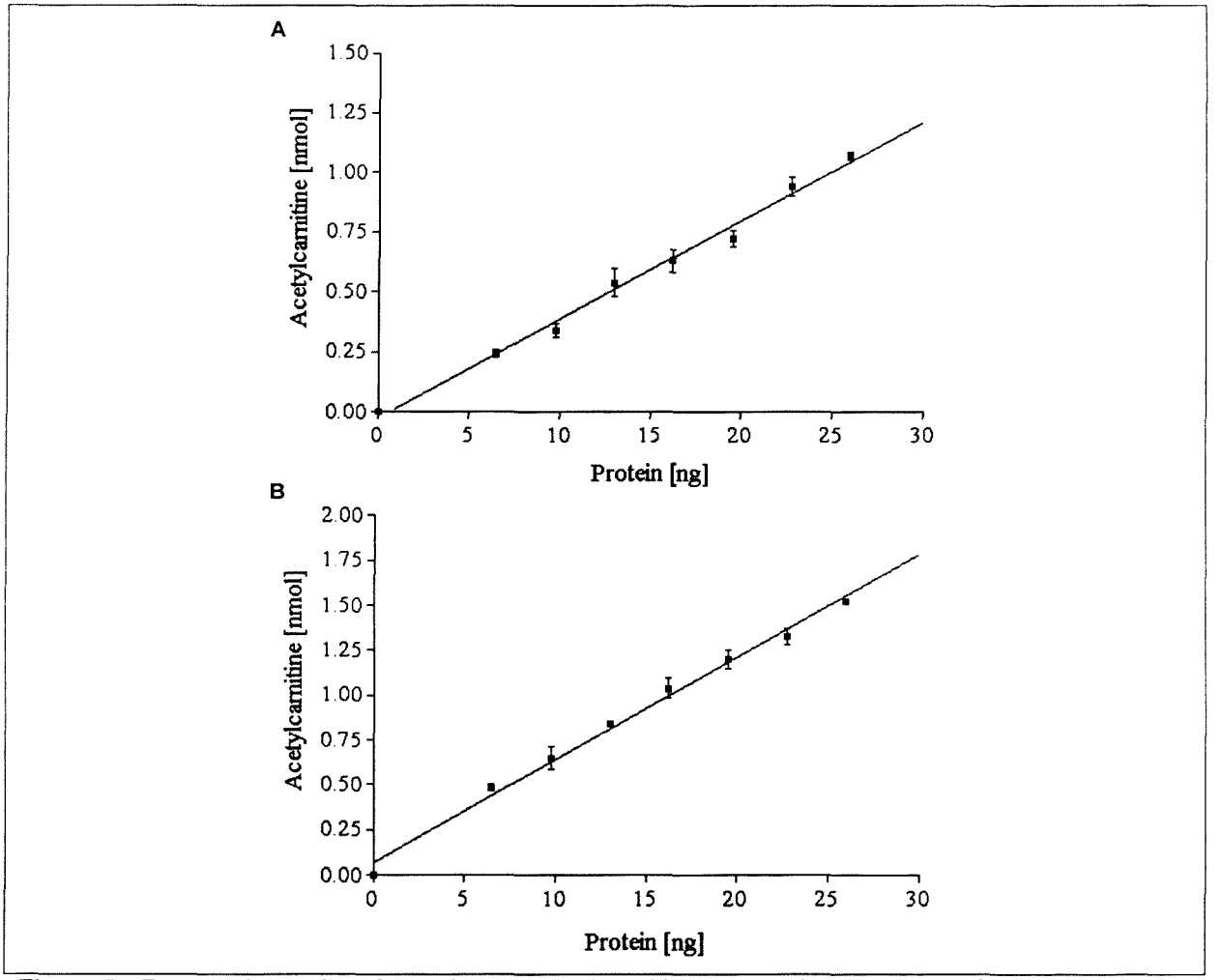

Figure 5. Protein-dependent formation of acetylcarnitine due to the presence of CAT enzymes in the yeast crude extract. The reaction was performed with different protein concentrations and was stopped either after 20 min (**A**) or after 40 min (**B**). The graph presents the average of six repetitions

5. Discussion

The yeast *S. cerevisiae* is an important model organism used to study molecular processes in eukaryotic cells. Due to the ease of genetic manipulation, this organism has contributed greatly to advancing the understanding of the molecular mechanisms involved in metabolic processes. In order to have a better understanding of the role of each of the CAT enzymes in the metabolism of yeast, it is important to study the activity of these enzymes collectively and individually in a wide range of physiological conditions. An assay was developed, based on the time-dependent formation of acetylcarnitine from free carnitine and acetyl-CoA. Acetyl carnitine was measured by HPLC in combination with tandem mass spectrometry.

Due to the presence of non-volatile salts in the incubation mixture, it was necessary to separate the acetylcarnitine from the more ionic compounds prior to detection in the mass spectrometer. Contrary to previously reported methods using MS/MS for the detection of acetylcarnitine, there is no extraction step of the assay samples used in this method. The HPLC step is sufficient for the removal of excess salts present in the assay samples. Simultaneous elution of the analyte and high concentrations of strong ionic compounds could suppress the ionisation of the analyte and negatively influence the lower limit of quantification. The inclusion of the separation step, accomplished by online HPLC on a C8 column, successfully sequestered acetylcarnitine for optimum sensitivity of detection. To optimise ionisation and detection, a low concentration of buffer was used and the samples were diluted by a factor of ten in acetonitrile, prior to separation. The low buffer concentration did not affect the buffering capacity of the enzyme assay mixture and the enzyme activity remained unaffected.

A high concentration of carnitine, used as precursor in the enzyme reactions, was also present in the reaction mixture. As indicated in Figure 2, carnitine and acetylcarnitine were not completely resolved by the HPLC system employed. The presence of carnitine, however, did not influence the detection and quantification of acetylcarnitine, due to the fact that different combinations of precursor and fragment ions were used in the detection of carnitine and acetylcarnitine. This demonstrates the inherent specificity of the multiple reaction-monitoring mode of detection and also provides the possibility of simultaneous quantification of both carnitine and acetylcarnitine in a single step.

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