Carnitine metabolism and biosynthesis in the yeast *Saccharomyces cerevisiae*

By

Jaco Franken
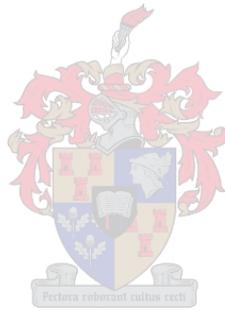
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Declaration

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SUMMARY

Carnitine plays an essential role in eukaryotic metabolism by mediating the shuttling of activated acyl residues between intracellular compartments. This function of carnitine, referred to as the carnitine shuttle, is supported by the activities of carnitine acyltransferases and carnitine/acylcarnitine transporters, and is reasonably well studied and understood. While this function remains the only metabolically well established role of carnitine, several studies have been reporting beneficial effects associated with dietary carnitine supplementation, and some of those beneficial impacts appear not to be directly linked to shuttle activity.

This study makes use of the yeast *Saccharomyces cerevisiae* as a cellular model system in order to study the impact of carnitine and of the carnitine shuttle on cellular physiology, and also investigates the eukaryotic carnitine biosynthesis pathway. The carnitine shuttle of *S. cerevisiae* relies on the activity of three carnitine acetyltransferases (CATs), namely Cat2p (located in the peroxisome and mitochondria), Yat1p (on the outer mitochondrial membrane) and Yat2p (in the cytosol), which catalyze the reversible transfer of activated acetyl units between CoA and carnitine. The acetylcarnitine moieties can be transferred across the intracellular membranes of the peroxisomes and mitochondria by the activity of the carnitine/acetylcarnitine translocases. The activated acetyl groups can be transferred back to free CoA-SH and further metabolised. In addition to the carnitine shuttle, yeast can also utilize the glyoxylate cycle for further metabolisation of in particular peroxisomally generated acetyl-CoA. This cycle results in the net production of succinate from two molecules of acetyl-CoA. This dicarboxylic acid can then enter the mitochondria for further metabolism. Partial disruption of the glyoxylate cycle, by deletion of the citrate synthase 2 (*CIT2*) gene, generates a yeast strain that is completely dependent on the activity of the carnitine shuttle and, as a consequence, on carnitine supplementation for growth on fatty acids and other non-fermentable carbon sources. .

In this study, we show that all three CATs are required for the function of the carnitine shuttle. Furthermore, overexpression of any of the three enzymes is unable to cross-complement deletion of any one of the remaining two, suggesting a highly specific role for each CAT in the function of the shuttle. In addition, a role for carnitine that is independent of the carnitine shuttle is described. The data show that carnitine can influence the cellular response to oxidative stresses. Interestingly, carnitine supplementation has a protective effect against certain ROS generating oxidants, but detrimentally impacts cellular survival when combined with thiol modifying agents. Although carnitine is shown to behave like an antioxidant within a cellular context, the molecule is unable to scavenge free radicals. The protective and detrimental impacts are dependent on the general regulators of the cells protection against oxidative stress such as Yap1p and Skn7p. Furthermore, from the results of a microarray based screen, a role for the cytochrome c heme lyase (Cyc3p) in both the protective and detrimental effects of carnitine is described. The requirement of cytochrome c is suggestive of an involvement in apoptotic processes, a hypothesis that is supported by the analysis of the impact of carnitine on genome wide transcription levels.

A separate aim of this project involved the cloning and expression in *S. cerevisiae* of the four genes encoding the enzymes from the eukaryotic carnitine biosynthesis pathway. The cloned genes, expressed from the constitutive *PGK1* promoter, were sequentially integrated into the yeast genome, thereby reconstituting the pathway. The results of a plate based screen for carnitine production indicate that the engineered laboratory strains of *S. cerevisiae* are able to convert trimethyllysine to L-carnitine. This work forms the basis for a larger study that aims to generate carnitine producing industrial yeast strains, which could be used in commercial applications.

OPSOMMING

Karnitien vervul 'n noodsaaklike rol in eukariotiese metabolisme deur die pendel van asiel residue tussen intersellulêre kompartemente te medieer. Hierdie funksie van karnitien heet "die karnitien-pendel" en word ondersteun deur verskeie karnitien asieltransferases en karnitine/asielkarnitien oordragsprotiëne. Die rol van die karnitien-pendel is redelik goed gekarakteriseer en is tot op hede die enigste bevestigde rol van karnitien in eukariotiese metabolisme. Verskeie onlangse studies dui egter op voordele geasosieer met karnitien aanvulling, wat in sommige gevalle blyk om onafhanklik te wees van die pendel aktiwiteit van karnitien.

Hierdie studie maak gebruik van die gis, *Saccharomyces cerevisiae*, as 'n sellulêre model sisteem om die impak van karnitien op sel fisiologie asook die eukariotiese karnitien biosintese pad te bestudeer. Die karnitien-pendel van S. Cerevisiae is afhanklik van die aktiwiteite van drie afsonderlike karnitien asetieltransferases (CATs), naamlik Cat2p (gelokaliseer in die peroksisoom en die mitochondria), Yat1p (op die buitenste membraan van die mitochondria) en Yat2p (in die sitosol). Die drie ensieme kataliseer die omkeerbare oordrag van asetielgroepe tussen CoA en karnitien. Die terugwaartse reaksie stel CoA-SH vry om sodoende verbruik te word in verdere metaboliese reaksies. Gis is in staat om, afsonderlik van die karnitien-pendel, gebruik te maak van die glioksilaat siklus vir verdere metabolisme van asetiel-CoA wat gevorm word in die peroksisoom. Gedeeltelike onderbreking van hierdie siklus deur uitwissing van die sitraat sintase (*CIT2*) geen, genereer 'n gisras wat afhanklik is van die funksie van die karnitien-pendel en ook van karnitien aanvulling vir groei op vetsure en nie-fermenteerbare koolstofbronne.

Hierdie studie dui daarop dat al drie CATs noodsaaklik is vir die funksionering van die karnitien-pendel. Ooruitdrukking van enige van die drie ensieme lei slegs tot self-komplementasie en nie tot kruis-komplementasie van die ander twee CATs nie. Hieruit word 'n hoogs spesifieke rol vir elk van die drie ensieme afgelei. 'n Pendel-onafhanklike rol vir karnitien word ook in hierdie werk uitgewys in die bevordering van weerstand teen oksidatiewe stres. Dit is noemenswaardig dat karnitien 'n beskermende effek het in kombinasie met oksidante wat ROS genereer en 'n nadelige effek in kombinasie met sulfhidriel modifiserende agente. Dit word aangedui dat karnitien antioksidant funksie naboots in die konteks van 'n gis sel terwyl die molekuul nie in staat is om vry radikale te deaktiveer nie. Beide die beskermende asook die nadelige inwerking van karnitien is afhanklik van Yap1p en Skn7p, wat reguleerders is in die algemene beskerming teen oksidatiewe stres. Die resultate van 'n "microarray" gebaseerde studie dui op 'n rol vir die sitokroom c heem liase (Cyc3p) in beide die beskermende en nadelige gevolge van karnitien aanvulling. Die vereiste vir sitochroom c dui op 'n moontlike rol vir apoptotiese prosesse. Hierdie hipotese word verder versterk deur 'n analise van die impak van karnitien op genoomwye transkripsievlakke.

'n Afsonderlike doelwit van hierdie studie was toegespits op die klonering en uitdrukking van die vier ensieme betrokke in eukariotiese karnitien biosintese in *S. cerevisiae*. Die gekloneerde gene, uitgedruk vanaf die konstitutiewe *PGK1* promotor, was geïntigreer in die gisgenoom om die pad op te bou. Die resultate van 'n plaat gebaseerde karnitien produksie toets dui aan dat die geneties gemanipuleerde gisrasse wel in staat is om trimetiellisien oor te skakel in L-karnitien. Hierdie werk vorm die hoeksteen van 'n studie wat die ontwikkeling van karnitien produserende kommersiële gisrasse as doelwit stel.

This dissertation is dedicated to Marieke

BIOGRAPHICAL SKETCH

Cornelius Jacobus (Jaco) Franken was born in Bloemfontein, South Africa, on 30 October 1976. He matriculated at the DF Malan High School, Bellville, in 1994. In 1995 he enrolled at Stellenbosch University and obtained a BSc degree in Biochemistry, Microbiology and Genetics in 1998. In 2000 he completed a BSc Hons degree and in 2003 a MSc in Wine Biotechnology at the Institute for Wine Biotechnology, University of Stellenbosch. In 2004 he enrolled for a PhD at the Institute for Wine Biotechnology.

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PREFACE

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the journal YEAST.

Chapter 1   **General Introduction and project aims**

Chapter 2   **Literature review**
The metabolic and physiological function of carnitine and the carnitine shuttle in yeast and higher eukaryotes.

Chapter 3   **Research results I**
Carnitine and carnitine acetyltransferases in the yeast *Saccharomyces cerevisiae*: A role for carnitine in stress protection.

Chapter 4   **Research results II**
General regulators of the oxidative stress response and cytochrome c are required for protective and detrimental effects of L-carnitine in *Saccharomyces cerevisiae*.

Chapter 5.   **Research results III**
Effect of carnitine supplementation on genome wide expression in the yeast, *Saccharomyces cerevisiae*.

Chapter 6   **Research results IV**
Reconstruction of the carnitine biosynthesis pathway from *Neurospora crassa* in the brewer's yeast *Saccharomyces cerevisiae*.

Chapter 7   **General discussion and conclusions**

CONTENTS

CHAPTER 5 EFFECT OF CARNITINE SUPPLEMENTATION ON GENOME WIDE EXPRESSION IN THE YEAST, *SACCHAROMYCES CEREVISIAE.*

CHAPTER 6. RECONSTRUCTION OF THE CARNITINE BIOSYNTHESIS PATHWAY FROM *NEUROSPORA CRASSA* IN THE BREWER'S YEAST, *SACCHAROMYCES CEREVISIAE.*

Chapter 1

General introduction and project aims

1.1. INTRODUCTION

Intracellular compartmentalization by biological membranes has contributed greatly to the evolutionary diversification of eukaryotes. It does, however, also create hurdles to the distribution and flux of many important metabolic pathways, since many intermediates of metabolism can not easily cross compartmental membranes. In the case of energy metabolism, such membranes are impermeable to CoA-activated acyl residues, the primary source of energy production in respiratory conditions. In yeast grown on non-fermentable carbon sources, the transfer of such residues is an essential requirement for energy production, since the generation and the further use of these metabolites take place in different compartments (Van Roermund *et al*, 1995). This obstacle is effectively circumvented by the reversible transfer of acyl residues from CoA to L-carnitine, catalysed by the activity of carnitine acyl transferases. Acylcarnitine can then be transported across the membranes of organelles by carnitine/acylcarnitine translocases. This system is conserved in function throughout the eukaryotic kingdom and is referred to as the carnitine shuttle.

In the yeast, *Saccharomyces cerevisiae*, the carnitine shuttle closely resembles that of higher eukaryotes with minor, but notable differences in composition. These differences can mostly be accounted for by distinctions in carbohydrate and fatty acid metabolism between yeast and higher eukaryotic organisms. Firstly, in yeast, β-oxidation of fatty acids occurs exclusively in the peroxisome, compared to peroxisomal and mitochondrial β-oxidation in mammals (Schmalix and Bandlow 1993; Stemple *et al*. 1998). Yeast also has access to a separate pathway, in the form of the partially peroxisomal glyoxylate cycle, producing succinate from acetyl-CoA, which can enter the mitochondrial tricarboxylic acid cycle for further metabolism. Consequently, yeast only displays carnitine acetyltransferase activity, whereas higher eukaryotes utilize several carnitine acyltransferases with variable affinities for acyl esters of varying chain lengths. The acetyl transferase activity in yeast is catalysed by three carnitine acetyltransferases (CATs), namely Cat2p (localized in both mitochondria and peroxisomes), Yat1p (localized on the outer-mitochondrial membrane) and Yat2p (residing in the cytosol) (Figure 1.1 A; Kispal *et al*. 1993; Schmalix and Bandlow 1993; Swiegers *et al*, 2001; Franken *et al*, 2008). In mammalian systems, a single carnitine acetyltransferase is present and active in both the peroxisomal and mitochondrial lumens. All three yeast CATs are required for a functional carnitine shuttle, but the specific roles of these

enzymes within the context of the shuttle and metabolism in general remain elusive (Swiegers *et al*, 2001). Obstructing the glyoxylate cycle by deletion of the citrate synthase 2 (*CIT2*) gene, which catalyzes the first reaction of this pathway, renders the yeast strain entirely dependent on the carnitine shuttle and also on carnitine supplementation for growth on fatty acids and non-fermentable carbon sources (Van Roermund *et al*, 1995; Swiegers *et al*, 2001). This also signifies that, unlike higher eukaryotes, which utilize four enzymatic steps to convert the precursor trimethyllysine to L-carnitine (Figure 1.1 B), *S. cerevisiae* is unable to neo-synthesize its own carnitine (Swiegers *et al*, 2002).
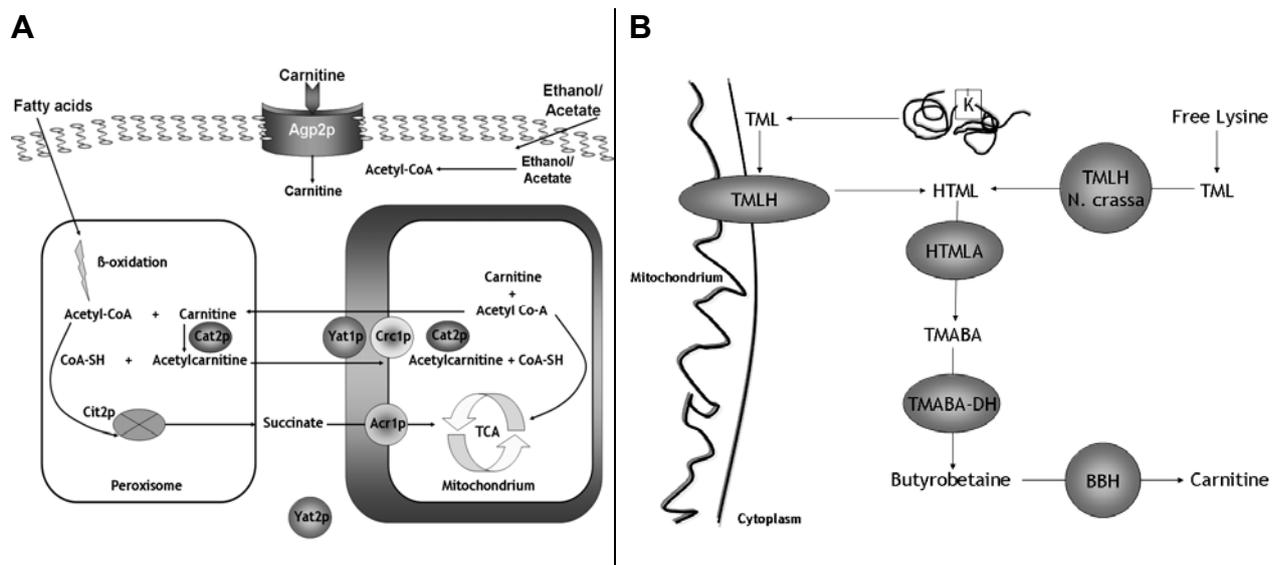
Figure 1.1. (**A**). Diagrammatic representation of the carnitine shuttle and glyoxylate cycle of the yeast, *Saccharomyces cerevisiae*. Indicated are the locations of the three yeast carnitine acetyltransferases namely Cat2p in the peroxisome and mitochondria, Yat1p on the outer mitochondrial membrane and Yat2p in the cytosol. The location of the carnitine/acetylcarnitine translocase in the mitochondrial membrane is also indicated. (**B**) Illustration of the carnitine biosynthesis pathway present in higher eukaryotes. The four central enzymes to this pathway, namely trimethyllysine hydroxylase (TMLH), hydroxytrimethyllysine aldolase (HTMLA), trimethylaminobutyraldehyde dehydrogenase (TMABA-DH) and γ-butyrobetaïne hydroxylase (BBH), catalyzes the conversion of trimethyllysine to L-carnitine. In mammals trimethyllysine originates as a product of protein degradation, whereas the fungus *N. crassa* is able to enzymatically convert free Lysine to L-carnitine (Vaz and Wanders, 2002).

The enzymes and activities of the carnitine shuttle in mammalian systems have been intensively studied and largely characterized throughout the second half of the previous century. The majority of current carnitine related research concentrates on the effect of systemic carnitine deficiencies and also the therapeutic applications of carnitine supplementation. Research has shown that carnitine supplementation is generally associated with beneficial effects in humans, and dietary supplementation of carnitine or acylcarnitines is proposed as either potential treatment or as supplemental treatment for a range of diseases (Ramsay *et al*, 2004; Calabrese *et al*, 2006; Petersen *et al*, 2005).

Among these, carnitine has been indicated to be of benefit to patients affected by cardiac ischemia, hepatic steatosis, type 2 diabetes, Alzheimer's disease and also as a supplemental treatment to counter the damaging effects of anti-retroviral administration and chemotherapies. The therapeutic effects of carnitine is mostly attributed to it's stimulatory function on mitochondrial metabolism and also on the balancing effect of the carnitine shuttle on the limited and compartmentalized pools of CoA and acyl-CoA's (Ramsay *et al*, 2004). Recent reports are, however, indicating possible roles for carnitine that would fall outside its metabolic function in the shuttling of the intermediaries of energy metabolism. Carnitine and acylcarnitines have been suggested to function as potentiators of the cells natural defenses against stress, as possible antioxidants and have also been indicated to have an effect on the regulation of programmed cell death (apoptosis) (Mutomba *et al*, 2000; Calabrese *et al*, 2006; Zhu *et al*, 2008; Wenzel *et al*, 2005; Ferrara *et al*, 2005). The precise mechanisms behind these effects are currently unclear and also hampered by difficulties of studying mammalian systems leading to contradictory reports, which may in part arise from differences between the systemic concentrations of carnitine achieved in separate studies. An overview of carnitine related metabolism and the impact thereof on eukaryotic cellular physiology comparing yeast and higher eukaryotes is presented in Chapter 2.

The use of yeast as a model system in the study of carnitine-related metabolism has aided in the initial description of the fates of cellular pools of acetyl-CoA and also in the description of shuttle components (Van Roermund *et al*, 1995; Kispal *et al*, 1993). However, with the shift of focus to more clinical application, the use of yeast in carnitine related research has diminished. Current knowledge of the shuttle's components and their function in yeast is lagging behind that of higher eukaryotic systems. Insights gained from using the well established genetic model system available in yeast cell biology may however contribute significantly to the understanding of the carnitine shuttle's function and also it's greater impact on cellular physiology. Therefore, a central aim of this work was to study the fundamental role and effects of the separate shuttle components, namely carnitine, acetylcarnitine and also the three CATs, in *S. cerevisiae*. As a means to achieve this, a phenotypic analyses of single, double and triple deletion mutants of the three yeast CATs and also the effect of carnitine supplementation in different stress conditions was undertaken. The results of this work are described in Chapter 3. An outcome of this study pointed towards a role for carnitine in the protection

against cellular stress induced by hydrogen peroxide and also certain organic acids. Since the impact of oxidative stress induced by various redox stressors have been extensively described in yeast, a follow-up study was pursued aiming to elucidate the mechanisms by which carnitine supplementation is able to protect against oxidative stresses. During the course of this work it became increasingly clear that the effect of carnitine under oxidative stress conditions could be mediated on a genetic level. On account of this, whole genome expression analysis, using cDNA microarrays were performed in order to screen for possible genetic links. A role for the cytochrome heme lyase (Cyc3p) in the mediation of the effect of carnitine in redox stress conditions was established in an initial analysis of these results. The results of this work are described in Chapter 4. Chapter 5 provides a more detailed description of the effects of carnitine on differential gene expression. The results indicate that the effect of carnitine supplementation is expected to have a direct impact on various aspects of cellular growth, iron homeostasis and possibly the regulation of programmed cell death.

A second part of this study involved the cloning of the four enzymes required for carnitine biosynthesis from the fungus, *Neurospora crassa,* and the reconstitution of this pathway in *S. cerevisiae*. *N. crassa* was chosen as a donor organism for the cloning of the carnitine biosynthesis genes, since it has also been indicated to have an enzymatic activity capable of converting free lysine to trimethyllysine, which serves as the precursor for this pathway (Borum and Broquist, 1977; Figure 1.1 B). This work forms part of a larger study, being conducted by SunBio at the University of Stellenbosch, of which the eventual aim would be to create an industrial strain of *S. cerevisiae* that would be able to biosynthesize carnitine. This work was done in collaboration with Dr. Anita Burger, from SunBio, and describes the establishment of this pathway in a laboratory yeast strain, forming part of the initial proof of concept for the project. The results of this work are discussed in Chapter 6.

1.2. PROJECT AIMS

The following aims were set for this project:

1. To investigate the function of L-carnitine and the carnitine shuttle using the yeast, *S. cerevisiae* as a genetic model system:
    (i)   Investigate the role of the components of the carnitine shuttle in *S. cerevisiae* using a genetic approach. .
    (ii) Investigate the protective function of carnitine in oxidative stress.

(iii) Establish *S. cerevisiae* as a model system for the elucidation of the metabolic and physiological functions of carnitine and the carnitine shuttle and specifically its effects on cellular stress.

2. Cloning and expression of genes involved in carnitine biosynthesis from the fungus, *N. crassa,* in *S. cerevisiae*:
    (i) Cloning of the four genes involved in carnitine biosynthesis from *N. crassa*.
    (ii) Reconstitution of the pathway in the yeast, *S. cerevisiae*.
    (iii) Establish if the recombinant strains of S. cerevisiae are able to neo-synthesize L-carnitine.

1.3. REFERENCES

Borum, P. R. and Broquist, H. P. (1977). Purification of S-adenosylmethionine: epsilon-N-L-lysine methyltransferase. The first enzyme in carnitine biosynthesis. *J Biol Chem* **252,** 5651-5.

Calabrese, V., Giuffrida Stella, A. M., Calvani, M. and Butterfield, D. A. (2006). Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes. *J Nutr Biochem* **17,** 73-88.

Ferrara, F., Bertelli, A. and Falchi, M. (2005). Evaluation of carnitine, acetylcarnitine and isovalerylcarnitine on immune function and apoptosis. *Drugs Exp Clin Res* **31,** 109-14.

Franken, J., Kroppenstedt, S., Swiegers, J. H. and Bauer, F. F. (2008). Carnitine and carnitine acetyltransferases in the yeast *Saccharomyces cerevisiae*: a role for carnitine in stress protection. *Curr Genet* **53,** 347-60.

Kispal, G., Sumegi, B., Dietmeier, K., Bock, I., Gajdos, G., Tomcsanyi, T. and Sandor, A. (1993). Cloning and sequencing of a cDNA encoding *Saccharomyces cerevisiae* carnitine acetyltransferase. Use of the cDNA in gene disruption studies. *J Biol Chem* **268,** 1824-9.

Mutomba, M. C., Yuan, H., Konyavko, M., Adachi, S., Yokoyama, C. B., Esser, V., McGarry, J. D., Babior, B. M. and Gottlieb, R. A. (2000). Regulation of the activity of caspases by L-carnitine and palmitoylcarnitine. *FEBS Lett* **478,** 19-25.

Palmieri, L., Lasorsa, F. M., Iacobazzi, V., Runswick, M. J., Palmieri, F. and Walker, J. E. (1999). Identification of the mitochondrial carnitine carrier in *Saccharomyces cerevisiae*. *FEBS Lett* **462,** 472-6.

Petersen, K. F., Dufour, S., Befroy, D., Lehrke, M., Hendler, R. E. and Shulman, G. I. (2005). Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* **54,** 603-8.

Ramsay, R. R. and Zammit, V. A. (2004). Carnitine acyltransferases and their influence on CoA pools in health and disease. *Mol Aspects Med* **25,** 475-93.

Schmalix, W. and Bandlow, W. (1993). The ethanol-inducible *YAT1* gene from yeast encodes a presumptive mitochondrial outer carnitine acetyltransferase. *J Biol Chem* **268,** 27428-39.

Stemple, C.J., Davis, M.A., Hynes, M.J. (1998). The *facC* gene of *Aspergillus nidulans* encodes an acetate-inducible carnitine acetyltransferase. *J Bacteriol* **180,** 6242-6251

Swiegers, J. H., Dippenaar, N., Pretorius, I. S. and Bauer, F. F. (2001). Carnitine-dependent metabolic

activities in *Saccharomyces cerevisiae*: three carnitine acetyltransferases are essential in a carnitine-dependent strain. *Yeast* **18,** 585-95.

Swiegers, J. H., Vaz, F. M., Pretorius, I. S., Wanders, R. J. and Bauer, F. F. (2002). Carnitine biosynthesis in *Neurospora crassa*: identification of a cDNA coding for epsilon-N-trimethyllysine hydroxylase and its functional expression in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **210,** 19-23.

van Roermund, C. W., Elgersma, Y., Singh, N., Wanders, R. J. and Tabak, H. F. (1995). The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. *Embo J* **14,** 3480-6.

Vaz, F. M. and Wanders, R. J. (2002). Carnitine biosynthesis in mammals. *Biochem J* **361,** 417-29.

Wenzel, U., Nickel, A. and Daniel, H. (2005). Increased carnitine-dependent fatty acid uptake into mitochondria of human colon cancer cells induces apoptosis. *J Nutr* **135,** 1510-4.

Zhu, X., Sato, E. F., Wang, Y., Nakamura, H., Yodoi, J. and Inoue, M. (2008). Acetyl-L-carnitine suppresses apoptosis of thioredoxin 2-deficient DT40 cells. *Arch Biochem Biophys* **478,** 154-60.

Chapter 2

LITERATURE REVIEW

The metabolic and physiological function of carnitine and the carnitine shuttle in yeast and higher eukaryotes.

2.1. INTRODUCTION

L-carnitine (3-hydroxy-4-*N*-trimethylaminobutanoate) is a quaternary amine derived from the amino acids lysine and methionine. The molecule owes its name to its initial discovery in extracts from meat at the beginning of the previous century. The central role played by carnitine in eukaryotic energy metabolism was, however, only recognized a half century later. Carnitine has, since then, been discovered in various microorganisms, fungi, plants and mammals (Bremer, 1983). Since most eukaryotes are able to catalyze the endogenous synthesis of L-carnitine, it has been classified as a conditionally essential nutrient.

The core metabolic function of carnitine is the transfer of acyl residues between the limited and compartmentalized pools of coenzyme A (CoA). The trafficking function of carnitine is assisted by various carnitine-acyltransferases, which catalyze the reversible trans-esterification of acyl groups to carnitine, and also integral membrane carnitine/acylcarnitine translocases (reviewed in Zammit, 1999 and Ramsey *et al*, 2004). This system of cooperative intra-organellar transport is referred to as the carnitine shuttle and has been extensively characterized in higher eukaryotes. A similar system has been elucidated and shown to function in a similar capacity in the yeast *Saccharomyces cerevisiae*. Several carnitine deficiencies, which can have severe metabolic effects, have been described and attributed to mutations of enzymes involved in the carnitine shuttle. Considering the central role of carnitine in energy metabolism and the modulation of free pools of CoA, several therapeutic avenues are currently being considered for diseases such as insulin independent (type 2) diabetes, obesity, steatoepatitis and lipotoxic heart damage (reviewed in Foster, 2004).

Several beneficial effects associated with carnitine supplementation have also been reported that can not be directly attributed to functions of carnitine within the context of the shuttle. These include reports indicating a role for carnitine in the defence against cellular stresses related to the build-up of reactive oxygen species, age associated mitochondrial decay and also apoptosis (Gulcin, 2006; Silva-Adaya *et al*, 2008; Hagen *et al*, 1998). In addition carnitine has recently been indicated to protect against oxidative and organic acid stress in *S. cerevisiae* (Franken *et al*. 2008). This review discusses the metabolic role of carnitine by in particular comparing yeast and higher eukaryotic systems and aims to identify focal areas where yeast research can contribute to the understanding of carnitine related impacts on cellular physiology.

2.2. SOURCES AND UPTAKE OF CARNITINE

2.2.1. CARNITINE DERIVED FROM THE EXRACELLULAR ENVIRONMENT

In humans, 75% of total carnitine is derived through dietary uptake of carnitine (Lennon *et al*, 1986; Stanley, 2004). The status of dietary carnitine intake in healthy humans correlates with carnitine plasma concentrations, which is exceptional for nutrients that are under tight metabolic regulation. The primary sources of dietary carnitine are animal products, especially red meats, which contains between 300 and 600 µmol of carnitine per 100 g. Smaller quantities are found in grain products (5 – 50 µmol/100g), fruits (5 – 20 µmol/100g), vegetables (5 – 20 µmol/100g), legumes (~ 0.5 µmol/100g) and dairy products (20 – 200 µmol/100g) (Rudman *et al*, 1977; Panter and Mudd, 1969). Although a small but statistically significant difference in carnitine plasma concentrations has been observed between people with an omnivorous diet compared to a cereal based diet or vegan diet, there is no evidence of clinical significance or pathophysiological consequences (Lombard *et al*, 1989; Cederblad and Lindsted, 1972; Cederblad 1987; Khan Siddiqui and Bamji, 1980). Dietary carnitine intake in higher eukaryotes is considered important but not essential, since endogenous biosynthesis is capable of compensating for deficiencies. Endogenous synthesis is observed in most, if not all, higher eukaryotes, and also in most fungi. However, the yeast *S. cerevisiae* is unable to neo-synthesize its own carnitine and is entirely dependent on extracellular sources (Swiegers *et al*, 2002). Furthermore, no information regarding carnitine concentrations within yeast are available.

2.2.2. CARNITINE BIOSYNTHESIS

2.2.2.1 Carnitine biosynthesis in higher eukaryotes

The carnitine requirement of higher eukaryotes can be met by endogenous synthesis. L-carnitine is synthesized via a four step enzymatic process, utilizing various hydroxylases and dehydrogenases, from the precursor trimethyllysine (for review see Vaz and Wanders, 2002). The pathway for carnitine biosynthesis was initially described and biochemically characterized in the fungus, *Neurospora crassa*, which utilizes the same central carnitine biosynthesis pathway conserved in higher eukaryotes (Fraenkel, 1954; Figure 2.1). A key difference between carnitine synthesis in *N. crassa* and mammalian

systems, however, is in the source of the precursor trimethyllysine. *N. crassa* possesses an enzymatic activity that sequentially methylates free lysine to form trimethyllysine (Borum and Broquist, 1977), whereas mammalian lysine is methylated only when part of a peptide. The mammalian system is therefore dependent on the liberation of the precursor after protein degradation (La Badie *et al*, 1976; Dunn *et al*, 1984).
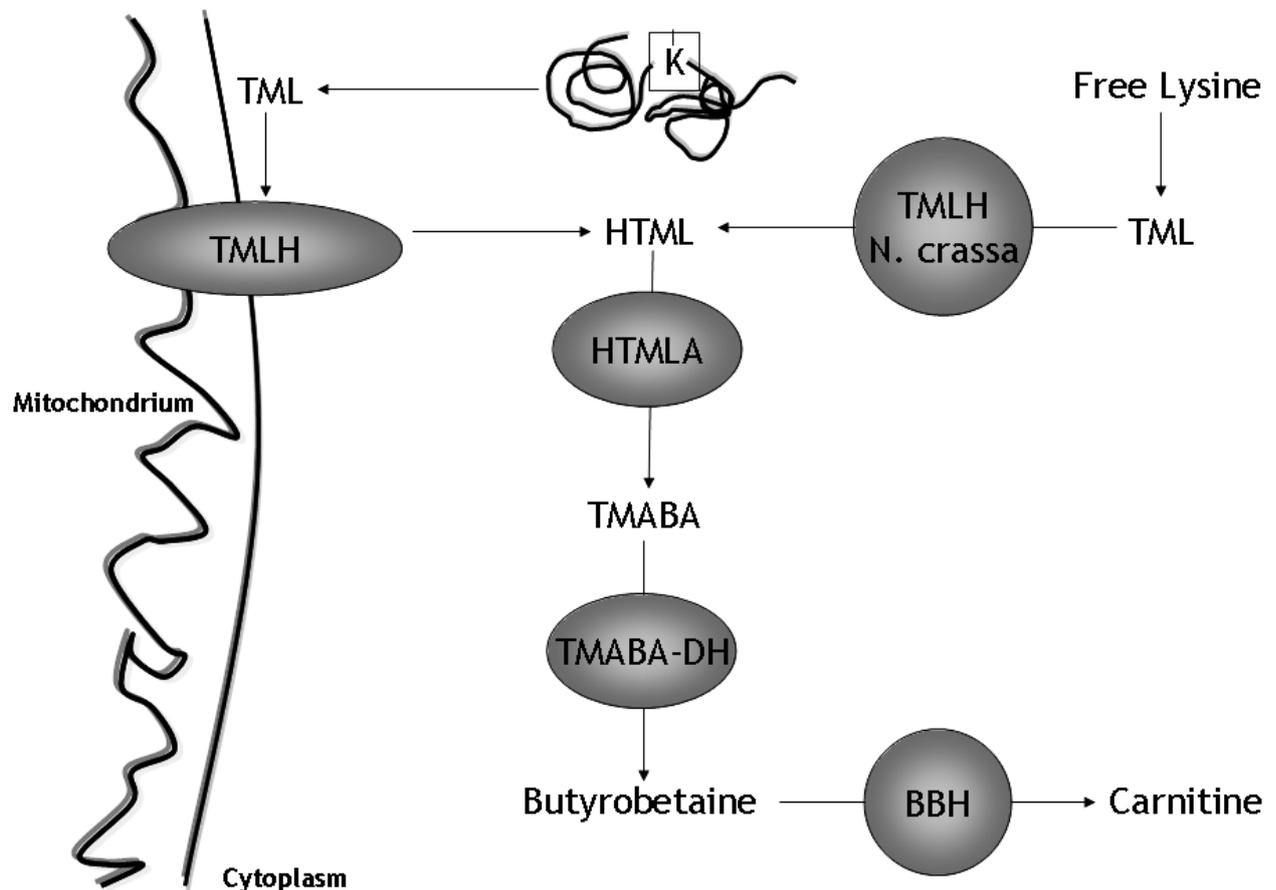
Figure 2.1. Diagrammatic representation of the eukaryotic carnitine biosynthesis pathway. The precursor, trimethyllysine (TML), originates either from protein degradation in mammals or via the enzymatic methylation of free lysine, as is the case in the fungus, *N. crassa*. TML is subsequently converted to L-carnitine by the enzymatic activities of trimethyllysine hydroxylase (TMLH), hydroxytrimethyllysine aldolase (HTMLA), trimethylaminobutyraldehyde dehydrogenase (TMABA-DH) and γ-butyrobetaïne hydroxylase (BBH). The intermediates of the pathway are indicated as follows, HTML = hydroxytrimethyllysine and TMABA = trimethylaminobutyraldehyde (Adapted from Vaz and Wanders, 2002)

The enzyme trimethyllysine hydroxylase (TMLH) catalyzes the first step of carnitine biosynthesis by the addition of a hydroxyl group to the third carbon of trimethyllysine, leading to the formation of 3-hydroxy-N^6-trimethyllysine (Hulse *et al*, 1978; Sachan and Broquist, 1980; Sachan and Hoppel, 1980). In mammalian systems the enzyme is localized in the mitochondria, compared to a cytosolic localization in *N. crassa*, and the conversion takes place in the liver, kidney, heart and brain. The enzyme requires 2-

oxoglutarate, Fe^{2+}, molecular oxygen and ascorbate as cofactors. In both *N. crassa* and mammals the pathway and enzymes downstream of the first reaction is located in the cytosol. The aldolytic cleavage of 3-hydroxy-N^6-trimethyllysine to form 3-hydroxy-N^6-trimethylaminobuteraldehyde is catalyzed by the enzyme hydroxytrimethyllysine aldolase (HTMLA), requiring pyridoxal 5'-phosphate as cofactor, to form 4-*N*-trimethylaminobutyraldehyde. The enzyme is active in most tissues, however, the greatest activity was found to be in hepatic cells (Hulse *et al*, 1978). The enzyme was purified from rat liver and found to be a serine hydroxymethyltransferase, which catalyzes a range of overlapping aldolytic reactions within the cell (Henderson *et al*, 1982; Stein and Englard, 1981). 4-N-trimethyllaminobutyraldehyde dehydrogenase (TMABA-DH) catalyzes the formation of γ-butyrobetaïne using niacin in the form of NAD as a cofactor (Vaz *et al*, 2000; Kikonyogo and Pietruszko, 1996; Lin *et al*, 1996; Kurys *et al*, 1993; Chern and Pietruszko, 1995). γ-Butyrobetaïne enters the circulatory system and is actively taken up, primarily by the liver and kidneys, where it is hydroxylated on the third carbon by the activity of γ-butyrobetaïne hydroxylase (BBH) in order to form L-carnitine (Vaz *et al*, 1998). Molecular oxygen and $Fe2^+$ are required for BBH activity. The synthesized L-carnitine is transported by the circulatory system to be taken up by other tissue cells.

Unlike mammals, *S. cerevisiae* is unable to neo-synthesize its own carnitine and none of the carnitine biosynthesis genes are encoded by the yeast's genome (Swiegers *et al*, 2001). Conversely, all four genes from the carnitine biosynthesis pathway have recently been identified and characterized in the yeast *Candida albicans* (Strijbis *et al*, 2009).

2.2.2.2. Heterologous expression of carnitine biosynthesis genes in *S. cerevisiae*

Recently, the *N. crassa* gene encoding TMLH has been cloned and functionally expressed in *S. cerevisiae* (Swiegers *et al*, 2002). In chapter 5, the cloning and expression in *S. cerevisiae* of the genes encoding the enzymes downstream of TMLH from *N. crassa* is described. In this study, the entire carnitine biosynthesis pathway was reconstituted in yeast and found to successfully catalyze the conversion of trimethyllysine to L-carnitine. In addition, the free lysine methyltransferase encoding gene was also cloned from the same organism and expressed in yeast. The assay system that was established to assess whether carnitine is produced, however, is not sensitive enough to indicate if this enzyme is functional and will require more detailed

analysis to establish if it is indeed functional. The same enzyme has previously been expressed in a bacterial system and found to catalyze the step-wise conversion of lysine to trimethyllysine (patent application No. WO 2007/007987 A1). Considering that carnitine is currently being marketed as a health supplement with an array of beneficial applications, yeast based, single cell or fermented products with enhanced carnitine levels could create substantial commercial interest.

2.2.3. CARNITINE UPTAKE

2.2.3.1. The mammalian organic cation transporters

The mammalian organic cation transporters belong to the major facilitator superfamily and are characterized by the presence of 12 transmembrane domains, as well as a large extracellular hydrophilic loop between the first and second predicted transmembrane domains which contains two to five glycosylation sites (reviewed by Lahjouji *et al*, 2001). A subfamily has been described which has the ability to transport carnitine and some of its esters. The members of the carnitine/organic cation transporter family, namely OCTN1, OCTN2 and OCTN3 have variable characteristics in their tissue specific expression profiles and also their affinities for carnitine.

OCTN1 was originally cloned from human fetal kidney cells and is expressed throughout a diverse range of tissues. It has been characterized as a multispecific, bidirectional, pH-dependent organic cation transporter (Tamai *et al*, 1997). The rat OCTN1 has a very low affinity for carnitine. Furthermore, carnitine transport is facilitated in a Na^+-independent manner by OCTN1 (Wu *et al*, 2000). Intriguingly, the mouse OCTN1 does exhibit Na^+-dependent carnitine transport, indicating an apparent specie-specific difference for the same transporter (Tamai *et al*, 2000). A second carnitine transporter, OCTN3 has been cloned from mice and was found to be expressed primarily in the testis and also kidney. OCTN3 mediates carnitine transport in a Na^+-dependent manner.

OCTN2 is considered to be the major transporter responsible for carnitine and also γ-butyrobetaïne (the direct precursor of carnitine in the biosynthesis pathway) uptake. OCTN2 functions as a Na^+-dependent carnitine transporter as well as facilitating Na^+-independent transport of other organic cations (Tamai *et al*, 1998). Na^+-dependent carnitine transport takes place at a high affinity (K_m = 4.3). In addition to carnitine, various organic cations and short chain acylcarnitine esters are also transported by

OCTN2. Furthermore, various clinically important drugs are transported by OCTN2, such as pyrilamine, quinidine, verapamil, and valproate (Wu *et al*, 1999). It has also been indicated that various xenobiotics, such as quinine and S-methylmethionine sulfonium significantly inhibit carnitine uptake by OCTN2. Carnitine uptake by OCTN2 was additionally shown to be inhibited by various ß-lactam containing antibiotics, namely cephaloridine, cefoselis, cefepime, and cefluprenam. Since OCTN2 is widely expressed in the heart, skeletal muscles, placenta, small intestines and the brain, absorption and dispersal of these drugs are likely to be effected by OCTN2.

A lack of functional OCTN2 carnitine transporters results in an autosomal recessive disease referred to as primary carnitine deficiency. Primary carnitine deficiency occurs at a frequency of between 1:40 000 – 1:100 000 (Koizumi *et al*, 1999; Wilcken *et al*, 2001). Several missense and nonsense OCTN2 mutations leading to residual carnitine transport activity have been identified (Lahjouji *et al*, 2001). The disease is characterized by a loss of 90-95% of systemic carnitine and has predominantly a metabolic, in the form of hypoglycemia or hyperammonemia, or cardiac presentation (Scaglia *et al*, 1998). In affected children, signs of metabolic disturbances usually manifest before the age of two and can result in coma and death if not treated in time with intravenous glucose. Cardiac presentation is more common in older patients in the form of cadriomyopathy. In some cases, children are only diagnosed due to the birth of an affected sibling and show only mild developmental augmentation (Wang *et al*, 2001). If primary carnitine deficiency is diagnosed before irreversible organ damage occurs, patients respond positively to dietary carnitine supplementation (100 – 400 mg/kg/day). The disease is diagnosed by the measurement of plasma carnitine levels and should be differentiated from other causes of carnitine deficiency, such as defects of fatty acid oxidation and the carnitine shuttle (Scaglia and Longo, 1999).

2.2.3.2. Additional transporters involved in mammalian carnitine uptake

In addition to the organic cation transporter family, CT2 and $ATB^{0,+}$ have also been shown to be involved in the uptake of carnitine (Enomoto *et al*, 2002; Nakanishi *et al*, 2001). CT2 was found to present in the epididymal epithelium of testis and not to be expressed in the brain or other tissues. $ATB^{0,+}$, which belongs to the Na^+, Ca^{2-} - dependent family of amino acid transporters, was also indicated to transport carnitine in an Na^+, Ca^- - dependent manner. $ATB^{0,+}$ was reported to be expressed in the intestinal tract, trachea, lungs, mammary glands and hippocampus (Sloan and Mager, 1999).

$ATB^{0,+}$, in combination with OCTN2 is considered to regulate carnitine uptake through the blood brain barrier (Ganapathy *et al*, 2000)

2.2.3.3. Carnitine uptake in *S. cerevisiae*.

The *S. cerevisiae* general amino acid transporter, Agp2p, was identified in a screen for mutants defective the carnitine-dependent transport of activated acetyl residues between the peroxisome and mitochondria (Van Roermund *et al*, 1995, 1999). *AGP2*, encodes for a protein of 596 amino acids wit 12 potential transmembrane domains and belongs to a family of assumed plasma membrane proton symporters (André *et al*, 1995). It was subsequently shown that Agp2p is required for the transport of carnitine into yeast cells. The data furthermore indicated that the transport of carnitine is Na^+-independent but H^+- dependent. The uptake of carnitine was also found to be induced in media containing oleate as carbon source, which could possibly be linked to a putative oleate response element (ORE) in the gene promoter and suggests carnitine uptake in yeast to be functionally coordinated with fatty acid metabolism. In a separate study, it was shown that carnitine uptake by Agp2p was shut down during conditions of osmotic stress (Lee *et al*, 2002). This effect was suggested to be due to the transcriptional repression of *AGP2* by elements of the Hog1 MAP kinase pathway. Yeast have been shown to import acetylcarnitine from the growth medium, however this uptake has not yet been linked to Agp2p mediated import (Franken *et al*, 2008). It would also be of interest to investigate the potential of the mammalian transporters to complement deletion of *AGP2* since this could provide a straightforward system in which to characterize various human, disease causing mutations.

2.3. THE CARNITINE SHUTTLE

2.3.1. THE CARNITINE SHUTTLE OF HIGHER EUKARYOTES

In mammals, β-oxidation of fatty acids takes place in both the peroxisome and mitochondria. Very long chain fatty acids are shortened or debranched in the peroxisome, resulting in only partial β-oxidation of fatty acids to acetyl-CoA or propionyl-CoA (from α-branched fatty acids) (Wanders *et al*, 1995; Leenders *et al*, 1996; Schulz 1991). For the complete oxidation of fatty acids to CO_2 the activated acyl groups from

the peroxisome need to enter the mitochondria (Bieber 1988; Reddy and Mannaerts 1994). The critical role of carnitine in the metabolism of fatty acids is due to the fact that acyl-CoA esters are impermeable to membranes of organelles and no transporter for these intermediates exists (for review see Zammit 1999). Trafficking between compartments is achieved by the transfer of acyl groups from CoA to carnitine by a diverse group of carnitine acyltransferases and transport across organellar membranes by the carnitine/acylcarnitine translocase (Figure 2.2). Aside from the inter-organellar transfer of activated acyl groups the carnitine shuttle has an additional role in the balancing of the cells compartmentalized and limited pools of coenzyme A. Various carnitine acyltransferases have been described, including the carnitine acetyltransferase (CAT, located in both the peroxisome and mitochondria), carnitine octanoyltransferase (COT, residing in the peroxisome), and the carnitine palmitoyltransferases (CPTI on the outer mitochondrial membrane and CPTII on the mitochondrial inner membrane). The carnitine/acylcarnitine translocase, CACT, is located in the inner membrane of the mitochondria. The activities of these enzymes and their function in the carnitine shuttle will be discussed separately in the following sections.

2.3.1.1. The carnitine acetyl (CAT) and octanoyl (COT) transferases

Carnitine acetyl transferases (CAT), using carnitine, acetylcarnitine, CoA-SH and acetyl-CoA as substrates, catalyze the freely reversible conversion between carnitine and acetylcarnitine. In addition the enzyme has also been shown to use other short chain acyl-CoA's, such as propionyl-CoA, as substrate. From studies in rat cellular systems, the subcellular distribution of the enzyme was shown to be both the in the peroxisome (30%), the lumen of the mitochondria (50%), and also in the lumen of the endoplasmic reticulum (ER) (20%) (Kahonen *et al*, 1979; Markwell *et al*, 1973). CAT is encoded by a single gene, with differential localization of the encoded proteins achieved by alternate mRNA splicing which leads to two transcripts, one of which contains a mitochondrial targeting sequence. Both peptides contain a putative peroxisomal targeting signal (AKL), suggesting that the presence of the mitochondrial targeting signal overrides the effect of the peroxisomal signal. It has been suggested that the "KVEL" sequence present in CAT could be responsible for ER targeting (Corti *et al*, 1994). CAT is an abundant protein that has been extensively studied. The chemical, kinetic and structural properties of the enzyme are well established and gene sequences have been identified

from many species, including yeast, human, mouse and rat (for review see Ramsey and Naismith, 2003).
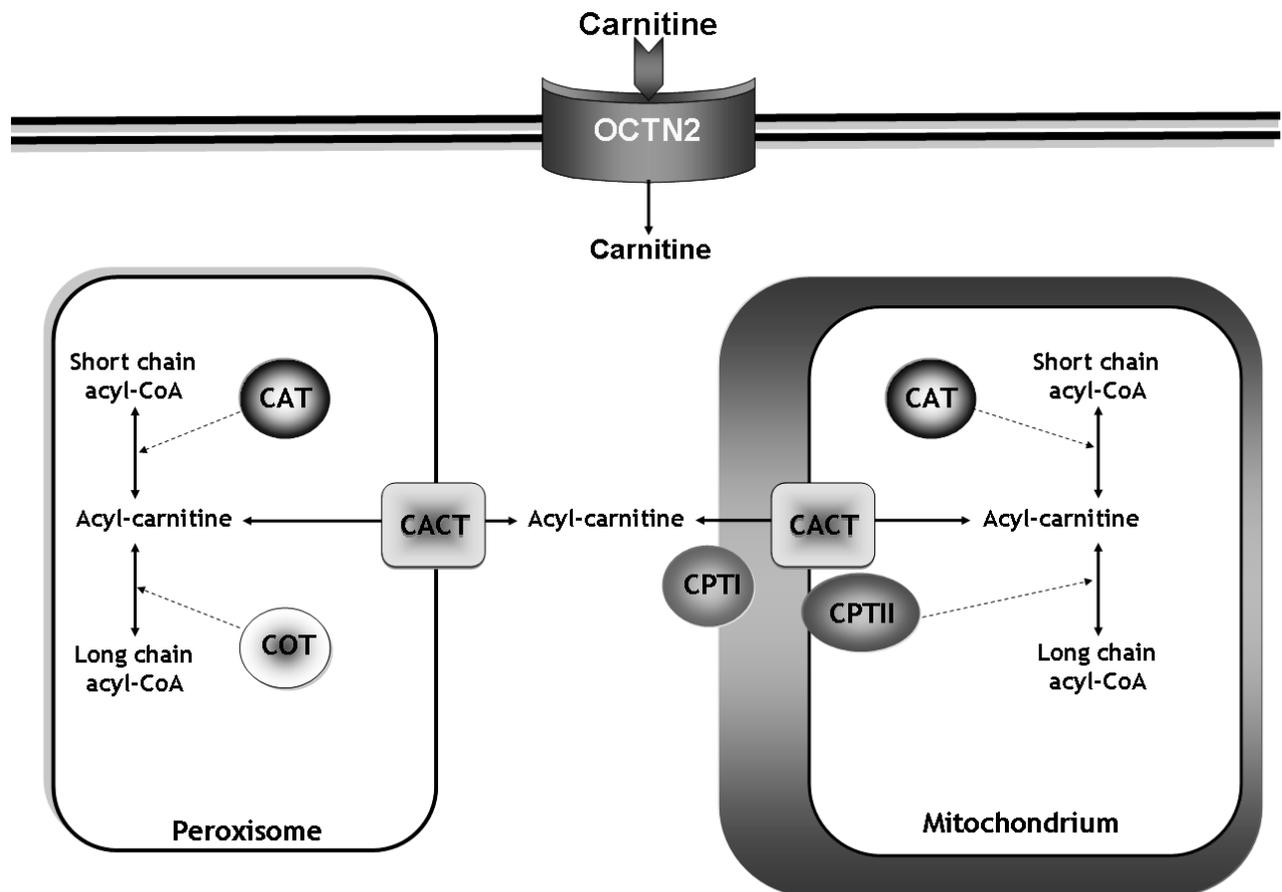
Figure 2.2. Localization of the different mammalian carnitine acyltransferases, carnitine acetyl transferase (CAT in the mitochondria and peroxisome), carnitine octanoyltransferase (COT in the peroxisome) and the two carnitine palmitoyltransferases (CPTI on the outer mitochondrial membrane and CPTII on the mitochondrial inner membrane).The carnitine/carnitine-acyl transporter CACT is located in both the mitochondrial and peroxisomal membranes. The diagram gives a simplified representation of the composition of the mammalian carnitine shuttle and its effect on the balancing of compartmentalized pools of CoA and acyl-CoA pools.

The function of peroxisomal CAT is the transfer of acetyl and propionyl moieties, generated by partial β-oxidation of fatty acids from CoA to carnitine, which is followed by transport out of the peroxisomes, allowing β-oxidation to proceed through regeneration of free CoA-SH. The acylcarnitine can than be transported to the mitochondria, enabling further metabolism. In the mitochondria CAT plays a central role in the regulation of acetyl-CoA metabolism, which lies at a metabolic crossroads between the catabolic TCA cycle, synthesis of molecules to be exported from the mitochondria for various cellular functions and the synthesis of ketone bodies in mammalian liver cells. The ratio between free CoA and acetyl-CoA plays a key role in the regulation of the switch

between glycolysis and gluconeogenesis and also the metabolism of fatty acids (for review see Zammit, 1994). The interconversion between carnitine and acetylcarnitine catalyzed by the activity of CAT is considered to have a major impact on the regulation of this decisive point in carbon metabolism. Furthermore, acetyl groups bound to carnitine provide a reservoir of activated acetyl groups that can be transferred to CoA and utilized as energy source through the citric-acid cycle in times of metabolic demand.

The carnitine octanoyl transferase (COT) enzyme is located in the peroxisomal matrix and catalyzes the transfer of medium to long chain acyl residues between CoA and carnitine. The enzyme's activity has a broad range of chain-length specificity, which would be a logical necessity since it is the only long chain acyl transferase present in the peroxisome and its activity would be required for the export of a wide range of acyl moieties (Ramsay, 1999).

2.3.1.2. The carnitine palmitoyltransferase system

Fatty acids in the cytosol are activated by the activity of the long-chain acyl-CoA synthase (LCAS), which is located on the outer mitochondrial membrane. The ATP released and stored after the β-oxidation of these residues represents a major energy source for most cells and tissues (Eaton *et al*, 1996; Kunau *et al*, 1995; Bartlett and Eaton, 2004). The activated acyl residues in the cytosol and peroxisomes utilize the carnitine palmitoyl system to enter the mitochondria for further metabolism (reviewed in Ramsay *et al*, 2001; Figure 2.3). This system consists of several proteins, including (i) the outer mitochondrial carnitine palmitoyltransferase I (CPTI) which converts acyl-CoA's to their representative acylcarnitine esters, (ii) the carnitine/acylcarnitine translocase (CACT) that translocates the produced acylcarnitines into the matrix of the mitochondria and (iii) the carnitine palmitoyl transferase II (CPTII), an enzyme associated with the inner leaflet of the mitochondrial membrane that converts the acylcarnitine esters to their respective acyl-CoA's. The carnitine palmitoyl transferase system plays a key regulatory role in controlling the flux through β-oxidation. As a consequence, mutations of either the carnitine palmitoyltransferases or the translocase result in potentially severe metabolic diseases. The carnitine palmitoyltransferases are also currently being considered as drug targets for the control of type 2 diabetes mellitus. The following section will discuss the function and regulation of this system and its components.
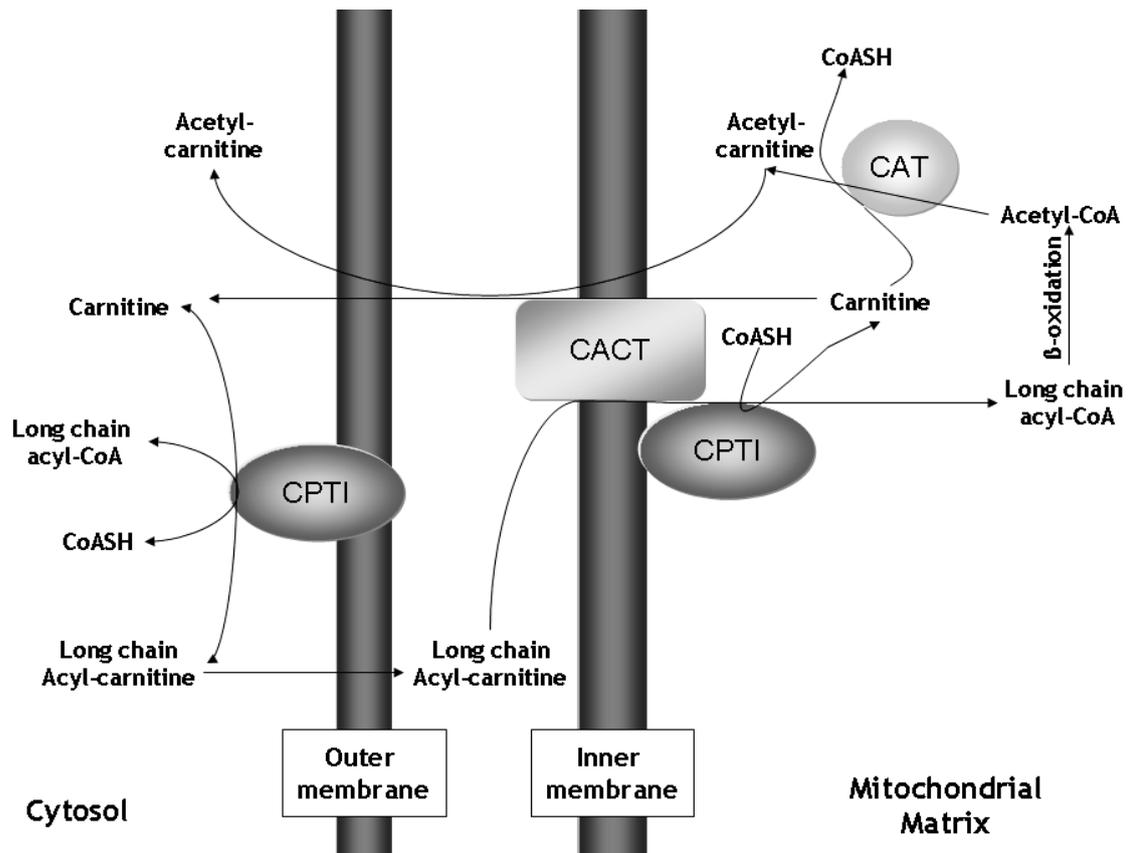
Figure 2.3. Diagrammatic representation of the carnitine palmitoyltransferase system, its effect on the regulation of the intramitochondrial acyl-CoA/CoA ratio and the locations of it's constituents within the mitochondria. Carnitine palmitoyltransferase I (CPTI), present on the outer membrane of the mitochondria, catalyzes the conversion of long-chain acyl-CoA's to long-chain acylcarnitines. The converted acylcarnitines are transported into the mitochondria by the activity of the carnitine/acylcarnitine translocase (CACT), to be reconverted to their represented acyl-CoAs by the activity of CPTII on the inner-mitochondrial leaflet. After β-oxidation, the resulting acetyl-CoA is converted to acetylcarnitine (by the activity of CAT) to be utilized in further metabolic processes (Adapted from Vaz and Wanders, 2002).

CPT I exists in three organ specific isoforms, namely the liver (L-CPTI), muscle (M-CPTI) and brain type (B-CPTI) carnitine palmitoyl transferases (McGarry and Brown, 1997; Price *et al*, 2002). The muscle and liver specific isoforms of CPTI differ significantly in their kinetic and regulatory properties. L-CPTI displays a higher affinity for carnitine and lower affinity for its physiological inhibitor malonyl-CoA compared to the muscle isoform. The two proteins are encoded by two separate genes, located on different chromosomes and also have distinct tissue distributions (McGarry and Brown, 1997; Kerner and Hoppel, 1998; Van der Leij *et al*, 2000). In contrast to CPTII, CPTI cannot be extracted in a catalytically active form and needs to be reconstituted in liposomes in order to recover activity when expressed in *Pichia pastoris* (McGarry and Brown, 2000). Mitoplast preparations from *S. cerevisiae* expressing CPTI provide an enzyme that has similar properties and membrane topology than the native form (Brown *et al*, 1994; Prip-Buus *et al*, 1998). Expression in yeast has considerable advantages,

since there is no CPT activity present in yeast, which would enable the study of isolated mutant forms of CPTI. The intrinsic dependence of CPTI activity on mitochondrial membrane fluidity has, nonetheless, provided difficulty in the characterization of CPTI's activity and the sensitivity to inhibitors (Zammit, 2008). The brain type CPTI, was initially considered to be inactive, since no activity could be detected from yeast extracts that heterologously expressed B-CPTI (Price *et al*, 2002). It has, however, been indicated that B-CPTI knock-out mice have reduced food intake and body weight, but do have an increased predisposition to obesity compared to wild type mice on a high-fat diet (Roomets *et al*, 2008). It was only recently indicated that B-CPTI does in fact have catalytic activity and that the protein is located in the endoplasmic reticulum (Sierra *et al*, 2008). In contrast, the mitochondrial matrix associated CPT II is present only as a single isoform and is ubiquitously expressed (Kopec and Fritz, 1973; West *et al*, 1971; Brown *et al*, 1993).

Acylcarnitines that are imported into the mitochondria by the carnitine/acylcarnitine translocase CACT do not equilibrate with acylcarnitine in the mitochondrial lumen (Murthy and Pande, 1985). Based on this finding it has been postulated that CPTII could be localized on the inner mitochondrial membrane in direct contact with CACT in such a manner that channeling would occur from the transporter into the receiving CPTII (Rufer *et al*, 2009). This would create a microenvironment from which the carnitine that is liberated, after transesterification to CoA, would be transported back to the cytosol by CACT. This creates in interesting possibility regarding the biochemical interaction between CACT and mitochondrial CAT. It has been observed that most hepatic CAT activity resides in the mitochondrial lumen, where it functions to buffer pools of activated acetyl-CoA by equilibration with acetylcarnitine (Ramsay and Naismith, 2003). In addition, excess acetyl-CoA generated by β-oxidation can be transported into the cytosol by CACT. This function is dependent on the transfer of acetyl groups from CoA to carnitine by CAT. If CPTII is involved in this process, as proposed by Rufer *et al.* (2009) the interaction between CPTII and CACT needs to be established in such a manner that bidirectional transport of acetyl-CoA to and from CPTII is possible. This proposed simultaneous processing, however, still needs to be experimentally supported. Such confirmatory findings would contribute to the understanding of the stimulation of gluconeogenesis by acetyl-CoA.

Malonyl-CoA was assumed to be mainly sourced from glycolysis, until studies using isotope labeled substrates established that peroxisomal β-oxidation is the major supplier

of acetyl-CoA for the synthesis of malonyl-CoA in heart cells (Poirier *et al*, 2002; Reszko *et al*, 2004). The inhibition of CPTI activity by malonyl-CoA, presents an interesting aspect of the regulation of fatty acid degradation. Detailed studies have identified the first 18 bp on the amino-terminal of CPTI to be required for malonyl-CoA inhibition (Shi *et al*, 1998; Shi *et al*, 1999). Moreover, an increase in membrane fluidity of the mitochondrial outer membrane disrupts the interaction between the N- and C-terminal domains, suggesting a degree of flexibility and complex mutual interactions between the two domains. In addition to malonyl-CoA sensitivity, phosphorylation based regulation of CPTI activity has also been suggested (Kerner *et al*, 2004; Kerner *et al*, 2005). Phosphorylation of CPTI on two sites, Ser741 and Ser 747, situated in the carboxy-terminal catalytic domain, has been associated with increased activity and modulation of malonyl-CoA sensitivity (Distler *et al*, 2007). Aside from phoshorylation it has been indicated that both the liver and muscle isoforms of CPTI is also nitrated (Fukumoto *et al*, 2002; Fukumoto *et al*, 2004). The addition of nitrate residues occurs on the C-terminal amino acids Tyr589 and Tyr282, which are speculated to have an impact on substrate binding. Acetylation of the N-terminal Ala2 has also been recently reported (Eaton *et al*, 2003). These modifications are likely to contribute to the alosteric regulation of CPTI activity.

CPTI, based on its key role in the maintenance of fatty acid β-oxidation and the connected effect on glucose homeostasis, has emerged as an attractive target in the treatment various metabolic diseases, such as type 2 diabetes, cardiac reperfusion injury and psoriasis. The modulation of CPTI activity by substances such as L-aminocarnitine, tetradecyl glycidic acid, etomoxir and phenylalkyl oxirane carboxylates received considerable research interest over the past five years. The discussion of this research area, however, falls outside the boundaries of this review (for recent reviews see Rufer *et al*, 2009)

2.3.2. THE CARNITINE SHUTTLE OF *S. CEREVISIAE*

The function of the carnitine shuttle is conserved between *S. cerevisiae* and mammals. There are, however, differences in the composition of the two systems that can be largely related to the variation in metabolic make-up when comparing yeast to higher
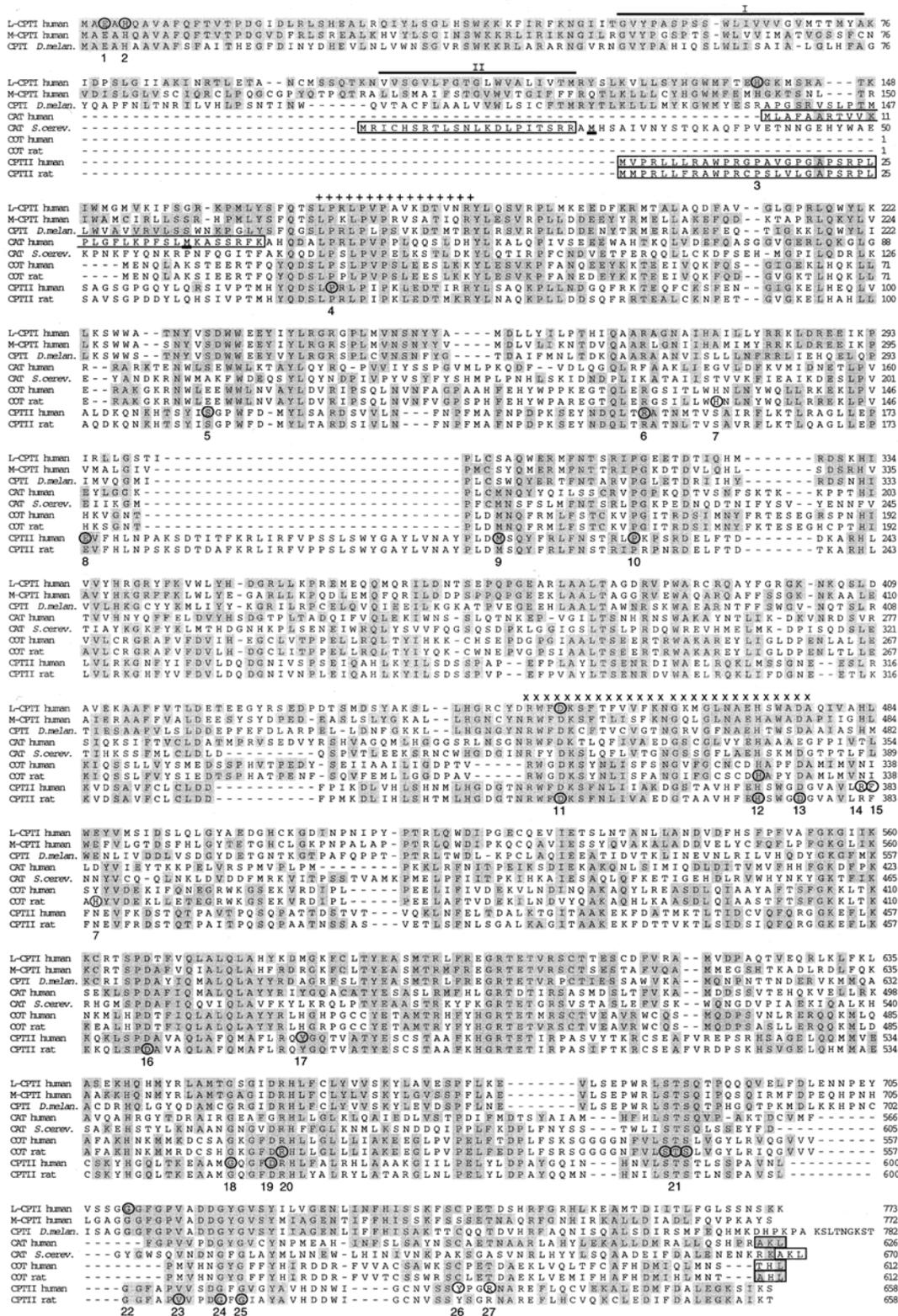
Figure 2.4. Cross-species sequence alignment of carnitine acyl-transferases. Shaded sequences indicate regions identical to a consensus sequence derived from the alignment of 15 carnitine acyltransferases. The Roman numbered bars indicates the two CPTI transmembrane domains. Carnitine acyltransferase domains are indicated using + and x symbols (Prosite PS00439 and PS0040). N-terminal mitochondrial and C-terminal peroxisomal targeting sequences are indicated in boxes. Start methionines of the peroxisomal forms of CAT are underscored. The circled and numbered residues indicate CPTI point mutations which result in loss of either malonyl-CoA sensitivity or enzyme activity (Ramsey *et al*, 2001).

eukaryotes. Firstly, β-oxidation in yeast takes place solely in the peroxisome, compared to mitochondrial and peroxisomal oxidation of fatty acids in mammals (Kunau *et al*. 1995). In addition, the generation of acetyl-CoA in the cytosol due to the metabolism of non-fermentable carbon sources does not occur in mammalian systems (Schmalix and Bandlow 1993; Stemple *et al*. 1998). Finally, an additional metabolic pathway that is absent in mammals, the glyoxylate cycle, impacts significantly on the metabolic importance of carnitine. This pathway allows the further metabolisation of peroxisomally generated acetyl-CoA without any requirement of the carnitine shuttle (Van Roermund *et al*. 1995). This metabolic "bypass" combines two molecules of acetyl-CoA to form succinate, which can then be transported by the membrane bound carrier Acr1p to the mitochondria (Palmieri *et al*. 1999). Deletion of the yeast citrate synthase (*CIT2*) gene, which is responsible for the first reaction of the glyoxylate cycle, effectively blocks this pathway and creates a yeast strain that is entirely dependent on the carnitine shuttle and carnitine supplementation for growth on non-fermentable carbon sources and fatty acids (Van Roermund *et al*. 1995; Swiegers *et al*, 2001). This finding has been efficiently used as a genetic tool for the isolation and characterization of the components of the carnitine shuttle in *S. cerevisiae*. Another significant difference between the two systems is the apparent absence of long chain carnitine acyl transferase activity in *S. cerevisiae* (Kispal *et al*. 1993). Indeed, to date, only carnitine acetyl-transferase activity has been described. However, this activity is catalyzed by three separate CATs in yeast. *CAT2* is considered to be the dominant enzyme of the three, responsible for 95% of total carnitine acetyl-transferase activity (Kispal *et al*. 1993). This enzyme, similar to the mammalian CAT (Figure 2.4), localizes to both the peroxisome and the mitochondria. The regulation of *CAT2* localization is achieved by the presence of two ATG codons in the gene's open reading frame and two separate transcripts, one of which encodes an N-terminal mitochondrial targeting signal. There is a peroxisomal targeting sequence (AKL) present at the C-terminal of both peptides and it appears that, similar to the mammalian CAT, the presence of the mitochondrial signal overrides the peroxisomal sequence (Corti *et al*, 1994). In addition to Cat2p, two additional CATs (Yat1p and Yat2p) which share a high degree of similarity have been identified (Schmalix and Bandlow 1993; Swiegers *et al*, 2001; Figure 2.5). Yat1p is associated with the outer mitochondrial membrane and Yat2p has been shown to be cytosolic (Franken *et al*, 2008). Interestingly, all three yeast CATs are required for a functional carnitine shuttle. Deletion of any one of the CATs in combination with *CIT2*

indeed results in complete loss of growth on non-fermentable carbon sources. Furthermore, over-expression of each CAT only results in self-complementation and not
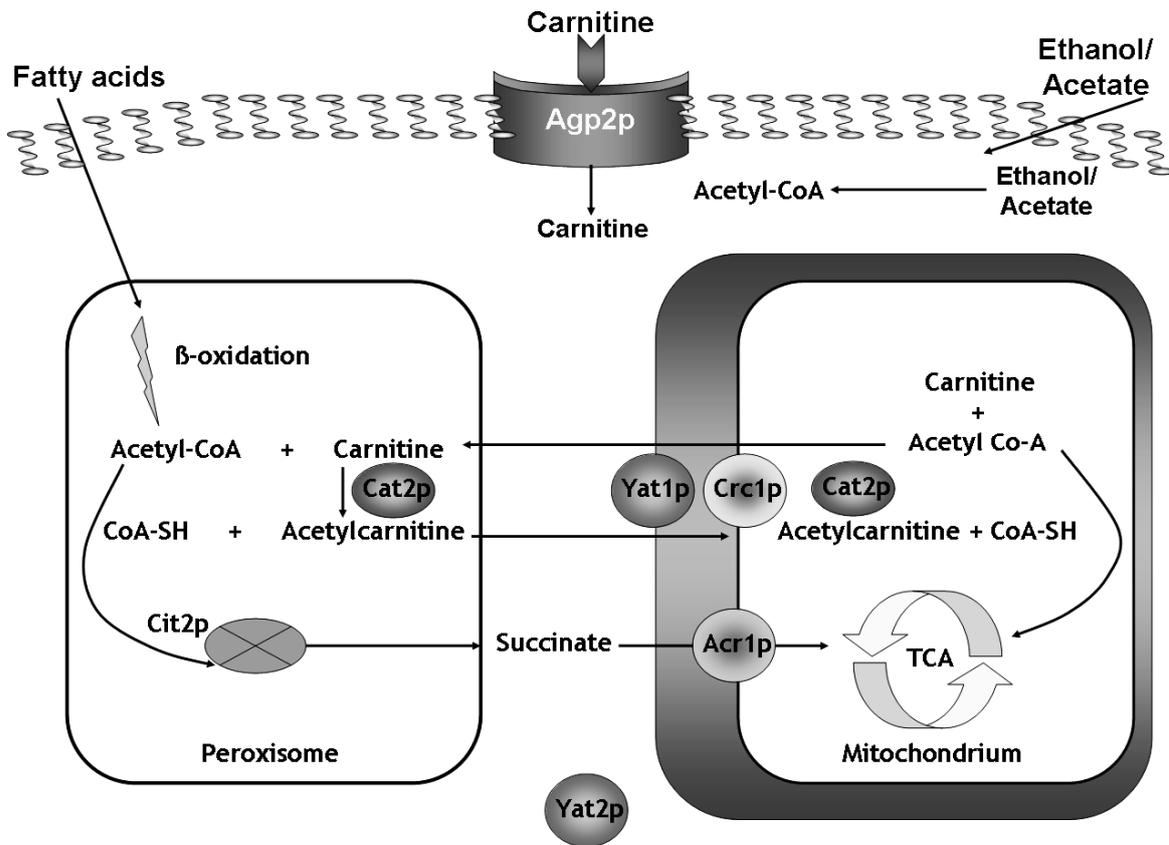
Figure 2.5. Diagrammatic representation of the carnitine shuttle and the glyoxylate cycle in *S. cerevisiae*. The three yeast carnitine acetyl transferases, Cat2p in the mitochondria and peroxisome, Yat1p on the outer-mitochondrial membrane and Yat2p in the cytosol are indicated along with the carnitine/acetylcarnitine translocase Crc1p. Cit2p combines two units of peroxisomally generated acetyl-CoA, the fist step of the glyoxylate cycle, forming succinate.

cross-complementation of any of the other two enzymes. This clearly indicates a very specific function for each of the three enzymes. It is currently not clear what the specific requirement of three separate CATs, all catalyzing the same reaction would potentailly be.

Apart from the three CATs, Crc1p, an orthologue of the human carnitine/acylcarnitine translocase CACT has also been identified. Transport of acetylcarnitine to the mitochondria is mediated by the activity of Crc1p (Palmieri *et al*. 1997; Van Roermund *et al*. 1995). It is, however, not clear if Crc1p is located in both the mitochondrial and peroxisomal membranes and if the transporter would be involved in the export of acylcarnitine residues from peroxisomes.

2.3.3. THE CARNITINE SHUTTLE OF *CANDIDA ALBICANS*

In the yeast *C. albicans*, phagocytosis leads to transcriptional profiles similar to that observed in cells growing on non-fermentable carbon sources (Lorenz *et al*, 2004). This finding has created a surge of interest in the processes involved in the regulatory mechanisms associated with carbon limitation in this pathogenic yeast. Among the group of genes significantly upregulated under these conditions are the carnitine acetyl-transferases (Prigneau *et al*, 2004). The genome of *C. albicans* encodes for three carnitine acetyl transferases, namely *CTN1*, *CTN2* and *CTN3*, which have been named according to their homology to the three *S. cerevisiae* CATs (*CTN1* is similar to*YAT1*, *CTN3* is similar to *YAT2* and *CTN2* is similar to *CAT2*). Similar to the *S. cerevisiae* *CAT2* gene, *CTN2* also has two separate start codons and conserved mitochondrial and peroxisomal targeting signals (Elgersma *et al*, 1995). Interestingly, *C. albicans* only possesses a mitochondrial and not a peroxisomal citrate synthase activity and is dependent on the carnitine shuttle for growth on fatty acids and non-fermentable carbon sources and also the transport of acetyl units from the peroxisome (Strijbis *et al*, 2008). Similar to *CAT2*, *CTN2* contributes the majority of CAT activity. The lack of a separate pathway for the channeling of peroxisomally generated acetyl-CoA would also explain that, in contrast to deletion of *S. cerevisiae* CATs, mutants of the *C. albicans* carnitine acetyl-transferases have distinct phenotypes when grown on different carbon sources (Zou and Lorenz, 2008). Both *CTN1* and *CTN2* are unable to grow on ethanol, acetate and citrate, whereas only *CTN2* is unable to grow on oleic acid as carbon source. *CTN3* has no distinguishable growth variances on separate carbon sources when compared to wild type (Prigneau *et al*, 2004; Zhou and Lorenz, 2008). Complementation of the *S. cerevisiae* CAT mutants results in intriguing differences and similarities between the two species. *CTN2* was found not to be functional in *S. cerevisiae*, whereas, *CTN3* was able to complement deletion of *YAT2* in combination with *CIT2*. On the other hand, *CTN1* was able to restore growth in both a Δ*cit2*Δ*yat1* and also a Δ*cit2*Δ*yat2* strain (Zhou and Lorenz, 2008).

2.4. PLEIOTROPIC CONSEQUENCES ASSOCIATED WITH CARNITINE-RELATED METABOLIC AVTIVITIES

In healthy humans, 80% of carnitine is present in its free form and the average ratio of acylcarnitine:carnitine is 0.25. A ratio of 0.4 is considered to be abnormal and indicative

of carnitine deficiency (Deufel, 1990). The ratio between serum acylcarnitine and free carnitine is highly sensitive to intramitochondrial metabolic alterations. A reduced pool of carnitine can either be a result primary carnitine deficiency (OCTN2 mutation) or has been attributed to a wide variety of diseases (Table 2.1). These clinical observations are in agreement with the view that a normal endogenous pool of carnitines is essential for normal cellular function and has provided the rational for the use of carnitine as a therapeutic supplement for the treatment of a wide variety of diseased or stressed states.

Table 2.1. Causes of carnitine deficiency.

Primary carnitine deficiency (OCTN2).

Secondary carnitine deficiency:
- Genetically mediated metabolic errors (fatty acid oxidation or branched chain amino acid metabolism disorders)

Acquired conditions:
- Decreased synthesis (liver cirrhosis)
- Decreased intake (malnutrition; malabsorption)
- Decreased body stores/increased requirement (sepsis; burns; trauma)
- Increased loss (heart failure; hypertension; diabetes)
- Mitochondrial dysfunction (HIV infection; inflammatory myopathies; chronic fatigue syndrome)
- Drugs (pivaloyl-antibiotics; valproate; antiretroviral nucleoside analogues)

As a consequence, substantial clinical evidence has accumulated over the past decade that supports a role for carnitine and its acyl esters as molecules with considerable therapeutic potential in a diverse group of diseases, such as Alzheimer's disease, heart ischemia, organic acidurias, and diabetes. The beneficial effects associated with carnitine supplementation can mostly be attributed to its function in the equilibration of the acylation state of the limited pool of CoA through the large cellular pools of carnitine. However, recent studies are indicating carnitine to have additional functions, unrelated to its metabolic role in the carnitine shuttle. In particular, links to the cells defense against stresses and also in the process of programmed cell death have been suggested (apoptosis) (Moretti *et al*, 1998; Mutomba *et al*, 2000; Pastorino *et al*, 1993). The metabolic and physiological effects associated with carnitine and acylcarnitine supplementation will be discussed in this section.

2.4.1. REMOVAL OF HARMFUL/EXCESS ORGANIC ACIDS

Acylcarnitine is exported along a concentration gradient from 0.5 - 1 mM inside the cytosol to 50 µM of total carnitine in the plasma (Sandor *et al*, 1985). The accumulation of excess intracellular acyl groups is reflected in the acylation state of the total plasma carnitine, which is clearly illustrated in the diagnostic acylcarnitine profiles of ß-oxidation impaired patients (Fingerhut *et al*, 2001; Sim *et al*, 2001; Gempel *et al* 2002). Diabetic patients excrete more long-chain acylcarnitine esters than control patients and this characteristic has been proposed to be used as a diagnostic tool in monitoring the therapy of diabetes mellitus (Moder *et al*, 2003). Carnitine and its short chain esters are effectively reabsorbed in the kidney while long chain variants are excreted (Evans and Fornasini, 2003). Therefore, sufficient carnitine levels are required in the plasma in order to excrete excess acyl moieties. Longer chain acylcarnitine esters can be excreted up to a total of one gram per day. In such cases, continuous carnitine supplementation is required to maintain the excretion of excess acids.

Deficiency of 2-methylacetoacetyl-CoA thiolase results in increased total and ester bound carnitine concentrations and also an enhanced acylcarnitine/free carnitine ratio. Supplementation of L-carnitine in these patients leads to an increase in the excretion of short and branched chain acylcarnitines (Fontaine *et al*, 1996). A similar effect is observed in the build-up of excess long-chain acyl groups is found in patients exhibiting a deficiency of the very long chain acyl-CoA dehydrogenase (VLCAD). Mice bearing knock-out mutations of VLCAD, unsupplemented with carnitine, displayed an increase in plasma acylcarnitines (C14-C18) and decrease in free carnitine. Exposing the fasted, VLCAD deficient mice to cold stress resulted in a further five-fold increase in the concentration of long chain acylcarnitines and resulted in a 33% mortality rate, indicating an increase in acylation of cellular CoA pools and the importance of maintaining the integrity thereof in cellular/organ function (Spiekerkoetter *et al*, 2004).

Certain drug treatments result in the production of excess organic acids, and the removal of these acids from cells appears dependent on the function of carnitine (for review see Arrigoni-Martelli and Caso, 2001). For instance, treatment of epilepsy with valproate leads to overacylation of the mitochondrial CoA pool and can result in the development of Reyes disease. Use of pivaloyl antibiotics also results in the excretion of excess acylated carnitine, with plasma free carnitine levels decreasing to almost 10% of the normal levels (Brass, 1994). Secondary carnitine deficiency arising from drug

treatment or from the dilution of plasma carnitine concentration observed in dialyses patients, results in high levels of ketone production, high blood acylcarnitine/free carnitine ratios and high lipid levels detectable in the liver and plasma (Steiber *et al*, 2004). These effects are effectively reversed by co-supplementation with carnitine.

2.4.2. MODULATION OF CARBON METABOLISM THROUGH THE CoA/ACYL-CoA RATIO

Cardiac ischemia is characterized by a relative deficit in the availability of myocardial oxygen. One of the strategies followed in the treatment of this disease is to optimize the function of the heart in relation to oxygen availability by improving the balance between fatty acid and pyruvate (from glycolysis) metabolism in the mitochondria (Lopaschuk, 2004). Increased rates of fatty acid oxidation are associated with lower rates of glucose oxidation and higher rates of glycolysis, which is considered to play a key role in myocardial ischemic injury (Stanley *et al*, 2005). When glucose is processed through glycolysis, pyruvate oxidation is inhibited, which results in an increase in lactate production and a decrease in intracellular pH that alters the movement of Ca^{2+} and Na^+ across the cell membrane and increases the ATP demand at a time when ATP formation is diminished (Dennis *et al*, 1991; Liu *et al*, 2002). As fatty acid oxidation recovers quicker during ischemia, glycolytic formation of lactate is increased, resulting in a decline in cardiac efficiency.

A common factor shared by pharmacological agents used in the treatment of ischemia and heart failure is the ability to drive energy metabolism in the direction of glucose oxidation by directly or indirectly activating pyruvate dehydrogenase (PDH) (Lopaschuk *et al*, 2002; Stanley *et al*, 2005). Using carnitine supplemented to the perfusion medium to obtain a two fold increase of carnitine in the myocardium resulted in an increased flux through PDH and subsequently also glucose consumption (Broderick *et al*, 1992). The effect of carnitine supplementation on PDH is mediated by the activity of the mitochondrial CAT. An increase in the intracellular carnitine concentration drives the forward reaction catalyzed by CAT toward the production of acetylcarnitine. As a consequence this is suggested to result in a decrease in mitochondrial acetyl-CoA, a potent activator of pyruvate dehydrogenase kinase, which keeps PDH in a more active state (Broderick *et al*, 1992; Figure 2.6 A). An unexpected outcome of carnitine supplementation in these conditions is a decreased rate of fatty acid oxidation. This counterintuitive action of carnitine seemingly results from the fact

that the lowered mitochondrial matrix concentration of acetyl-CoA overcomes the mass-action effect of an increased carnitine concentration on CPTI activity (Saddik *et al*, 1993). This would result in an increased flux through the electron transport chain, associated with a higher rate of pyruvate oxidation. The beneficial effect of an increased rate of glucose oxidation relative to fatty acid oxidation on cardiac function has
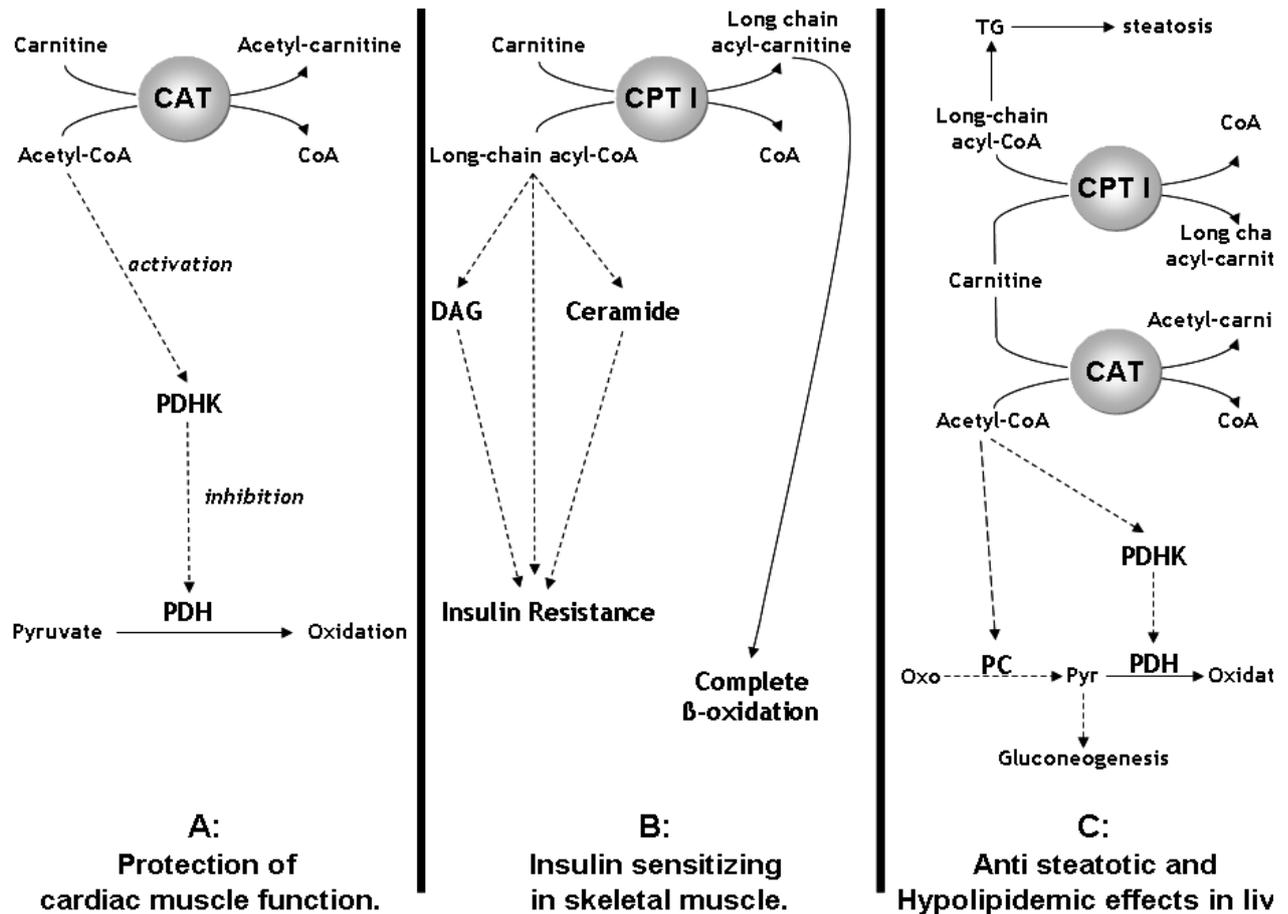
Figure 2.6. Possible effects of supplementation of high levels of carnitine. Direction of flux is indicated by solid arrows and broken arrows indicate regulatory effects (**A**) Increased carnitine concentration affects the use of carbon fuel sources in cardiac muscle. The mass-action effect caused by high levels of carnitine on CPTI is overcome by the effect of carnitine that shifts the equilibrium of the mitochondrial CAT away from acetyl-CoA synthesis. The resulting de-inhibition of pyruvate dehydrogenase kinase (PDHK) results in enhanced oxidation of glycolytic pyruvate. (**B**) Insulin sensitivity is affected by fatty acid oxidation rate. The major contribution towards systemic insulin-sensitive glucose metabolism resides in the skeletal muscles. Derepressed fatty acid oxidation, increasing the supply of long-chain acyl-CoA for diacylglycerol (DAG) and ceramide synthesis, and also incomplete fatty acid oxidation, resulting in carnitine esters of β-oxidation intermediates, have been suggested to influence insulin resistance. (**C**) Hepatic steatosis is highly related to insulin resistance. Carnitine supplementation potentially increases fatty acid oxidation, diverting metabolism away from triacylglyceride (TG) synthesis, through a mass-action effect on the reaction catalyzed by CPTI. Intermediates of TG synthesis are known to result in increased insulin resistance in the liver and also other tissues. High levels of carnitine shifts the CAT catalyzed reaction away from acetyl-CoA synthesis, resulting in lower levels of activated pyruvate carboxylase (PC) and PDH kinase, which leads to derepressed gluconeogenesis. (Adapted from Arduini *et al*, 2008).

been well established (Broderick *et al*, 1992). Propionyl-carnitine has also been indicated to have the same beneficial effects, with the added benefit of ATP generation from the metabolism of the propionyl moiety (Wiseman and Brogden, 1998; Zammit *et al*, 1998; Schonekess *et al*, 1995; Loster *et al*, 1999; Felix *et al*, 2001). As can be expected, acetylcarnitine did not show the same beneficial outcome on cardiac function, since it would not have a similar effect on the mitochondrial pools of acetylcarnitine and acetyl-CoA.

Carnitine supplementation has also been indicated as a possible effective treatment of the metabolic inflexibility and insulin insensitivity associated with type 2 diabetes (Gunal *et al*, 1999; Biolo *et al*, 2008). Skeletal muscle is a major site of glucose deposition in response to insulin and as a result a major site of insulin resistance in type 2 diabetes. A major contributing factor of insulin resistance in skeletal muscle appears to be a decreased ability of insulin to induce the switch from lipid to carbohydrate oxidation (Kelley and Mandarino, 2000). Interestingly, a 15 % increase of skeletal muscle carnitine was shown to result in a significant decrease in PDH activity (30%) (Stephens *et al*, 2006). The carnitine treatment additionally resulted in a 30 % increase of glycogen and a 40 % decrease of muscle lactate content. These observations suggest that an increase in muscle carnitine concentration reduces glycolytic flux and carbohydrate oxidation at the level of PDH, by diverting muscle glucose uptake towards glycogen storage, resulting from an increase in non-oxidative glucose disposal (Stephens *et al*, 2007). As can be expected from the reciprocal relationship between fatty acid oxidation and glucose oxidation, carnitine administration leads to an increase in the oxidation of fat, an effect which has been observed in healthy and overweight patients (Müller *et al*, 2002; Wutzke and Lorenz, 2004). Comparing the effect carnitine supplementation in cardiac to skeletal muscle illustrates a biphasic function, which would depend on whether the mass-action effect on CPTI or the changes in intramitochondrial acetyl-CoA concentrations, resulting from mass-action effect on the mitochondrial CAT, would predominate. Alternatively, it is also possible that increased levels of carnitine initiate distinct actions depending on the relative importance of the equilibriums catalyzed by the two carnitine acyltransferases in cardiac compared to skeletal muscle cells.

Administration of carnitine may also result in the alleviation of the impairment of the insulin signaling cascade, associated with type 2 diabetes patients, that functions in skeletal muscle cells. It is well known that an increased lipid supply to skeletal muscle

leads to an accumulation of long chain acyl-CoA esters, diacylglycerols and ceramides, which are potent inhibitors of insulin signaling (Kelley and Mandarino, 2000; Schmitz-Pfeifer, 2000; Summers, 2006). The stimulatory mass-action effect of increased levels of carnitine on the activity of CPTI may lower long chain acyl-CoA levels, thereby improving insulin signaling (Ramsay and Arduini, 1993; Zammit, 1999; Figure 2.6 B).

When considering diseases such as insulin resistance, type 2 diabetes, dyslipidemia and end-stage renal disease, which are affected by alterations in lipid and glucose homeostasis, the liver is known to play a central role in the progression of such metabolic disorders. The plasma lipid profiles of patients suffering from such diseases are often characterized by high levels of triglycerides and low levels of high density lipoprotein (HDL) cholesterol (Prichard, 2003; Liu and Rosner; 2006; Kwan *et al*, 2007). Carnitine supplementation in this context should either result in a decrease in *de novo* fatty acid synthesis in the liver by diverting acetyl moieties to the formation of acylcarnitine, which would subsequently be secreted, or by reducing the availability of long chain acyl-CoAs, which serve as a substrate for triglyceride synthesis (Colemen and Lee, 2004; Figure 2.6 C). This action would be mediated by the mass-action effect of high levels of carnitine on the carnitine acyl-transferases, CPTI and the mitochondrial CAT. Consequently, high levels of supplemented carnitine would be predicted to result in a lower secretion rate of very low density lipoprotein (VLDL) triglyceride and associated cholesterol, translating in a reduction of plasma lipids (Coleman and Lee, 2004). Lowered secretion of VLDL would in turn lead to an increase in plasma HDL, since reducing the amount of triglyceride present in HDL decreases its catabolism in the liver (Marsh *et al*, 2000). Moreover, it has also been suggested that carnitine may be required for the transfer of long-chain acyl groups to the endoplasmic reticulum for the esterification of diacylglycerol to form triacylglycerol destined for excretion within VLDL (Zammit, 1999). It is also considered likely that the increase of fatty acid oxidation by supplemented carnitine would antagonize the development of hepatic steatosis, which contributes greatly to the susceptibility of insulin resistance on a systemic level (Petersen *et al* 2005, Arduini *et al*, 2008).

While these hypotheses have been substantiated in some studies (Power *et al*, 2007; Rajasekar and Anuradha, 2007; Rodrigues *et al*, 1988), other studies showed no significant effect of carnitine supplementation in animal models of insulin resistance and dyslipidemia (Brady *et al*, 1986; Dai *et al*, 2004). The discrepancies in the datasets can probably be explained by variations in the concentration of supplemented carnitine and

the resulting concentrations that were achieved in plasma and target organs. The pharmacological actions exerted by carnitine are at carnitine concentrations (low millimolar range) higher than the low micromolar to low millimolar range systemic and cellular levels that are normally present (Rebuoche and Engel, 1984; Evans, 2003; Evans and Fornasisni, 2003; Tein, 2003). The maintenance of high carnitine plasma and target organ levels that are required to illicit such desired pharmacological responses remain difficult to achieve. Achieving such levels would be required to obtain more conclusive data to resolve these questions. At this stage, the most effective means of delivering and maintaining high systemic levels of carnitine currently seems to be through peritoneal or hemodialysis (Vacha *et al*, 1983; Vacha *et al*, 1989; Veselá *et al*, 2001; Brass *et al*, 2001).

Since the maintenance of specific carnitine concentrations may be less of a hurdle in yeast biology, a better understanding of the specific functions of the three yeast CATs and their influence on the regulation of carbon metabolism through modulating the concentrations of compartmentalized pools of CoA would surely contribute to the understanding of the cellular role of the carnitine shuttle. It would specifically be of interest to establish the impact of the two "minor" CATs, Yat1p and Yat2p in this regard.

2.4.3. MODULATION OF THE CELLULAR STRESS RESPONSE

Several studies are proposing a role for acetylcarnitine in the prevention of the deterioration of brain function associated with aging and neurodegenerative disorders. Acetylcarnitine is more prevalently used in the study of neurodegeneration since it is readily able to cross the blood-brain barrier (Parnetti *et al*, 1992). The beneficial effect of acetylcarnitine supplementation in neurodegeneration is mainly due to its stimulation of mitochondrial respiration, allowing the neuronal ATP production required for maintenance of membrane potential (McDaniel *et al*, 2003). Acetylcarnitine has, however, been indicated to be neuroprotective by a variety of other effects such as by stimulating an increase in protein kinase C (PKC) activity. Supplementation of acetylcarnitine has also been shown to counteract the loss of NMDA receptors and increasing the production of neurotrophins, effects that are exclusively related to synaptic plasticity (McDaniel *et al*, 2003).

In recent studies, it has been demonstrated that acetylcarnitine, by transcriptional induction of heme-oxygenase-1 (HO-1) and Hsp70, is able to reduce Aβ toxicity in

primary cortical neuronal cultures (Abdul *et al*, 2006). Studies in rats have shown an improvement of life-span and cognitive behavior and also long-term memory (McDaniel *et al*, 2003). Moreover, long-term acetylcarnitine administration prevents the age related decay of mitochondrial respiration and decreases oxidative stress markers by the up-regulation of HO-1, Hsp70 and superoxide dismutase-2 (SOD2) in ageing rats (Calabrese *et al*, 2006b). Acetylcarnitine has been shown to induce HO-1 expression in a time and dosage dependent manner. This effect is associated with the upregulation of other heat shock proteins and also the redox sensitive transcription factor Nrf2. It has been proposed by the authors of this work that the upregulation of HO-1 and Hsp's might involve the acetylcarnitine dependent acetylation of DNA binding transcription factors, such as Nrf2, resulting in the induction of target genes (Calabrese *et al*, 2005; Calabrese *et al*, 2006a) (Figure 2.7). This hypothesis, however, still needs to be substantiated. It could, nevertheless, suggest a role for acetylcarnitine as a potentiator of the cells natural defense response and would present promising therapeutic possibilities in pathophysiological conditions where the activity of HO-1 pathway is required.
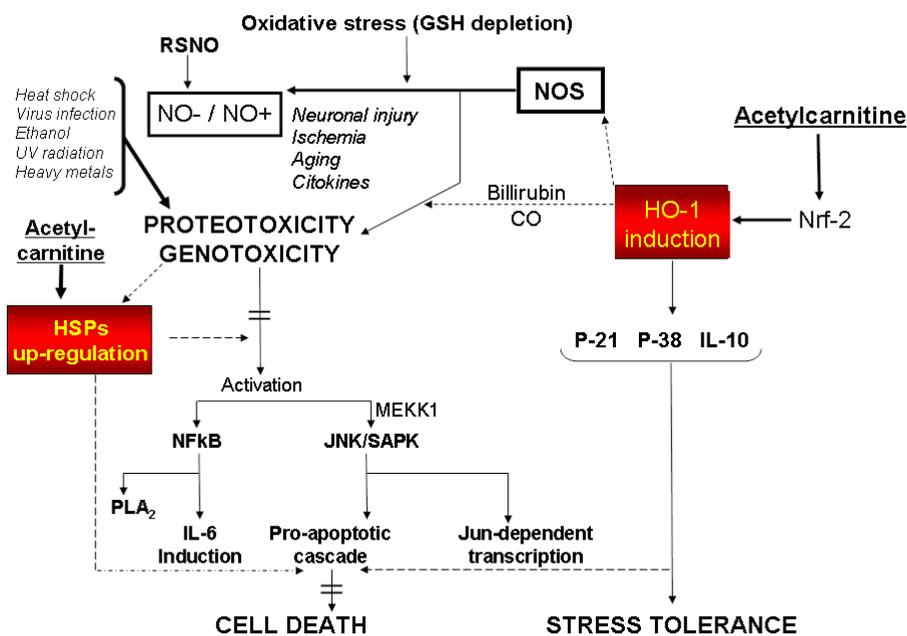
Figure 2.7. The proposed role of acetylcarnitine in the regulation of the cellular stress response. Depletion of heat shock proteins resulting from various proteotoxic and genotoxic events leads to the induction of stress kinase, and pro-inflammatory and apoptotic signaling cascades. Stress induced apoptosis is prevented by Hsp70, which interferes with SAPK/JNK signaling and blocks the caspase proteolytic cascade. By activating the transcription factor, Nrf2, acetylcarnitine induces the upregulation of heme oxygenase-1 (HO-1) and Hsp60 and could counteract nitrosative stress and NO-mediated toxicity. HO-1 may also directly decrease NO synthase protein levels by degrading heme, which serves as a co-factor. (PLA2: phospholipase; IL: interleukin; SAPK: stress-activated protein kinase; JNK: c-Jun N-terminal kinase; GSNO S-nitrosoglutathione; adapted from Calabrese *et al*, 2006)

2.4.4. MODULATION OF PROGRAMMED CELL DEATH

Signal transduction via the cell surface glycoprotein Fas, also referred to as CD95, is considered central in the regulation of programmed cell death (apoptosis). L-carnitine was shown to inhibit apoptosis by interaction with Fas-ligand and the Fas receptor systems (Moretti *et al*, 1998). Fas receptor mediated signal transduction activates acid sphingomeylinase in lysosomes and consequently the breakdown of sphingomyelin and the release of ceramide occur. The addition of carnitine results in an immediate inhibition of acid sphingomeylinase under in vivo and in vitro conditions (Di Marzio *et al*, 1997). In addition carnitine was shown to inhibit caspase 3, 7 and 8 and also the mitochondrial permeability transition (Mutomba *et al*, 2000; Pastorino *et al*, 1993). In T lymphocytes a separate mechanism was described for the inhibition of apoptosis by carnitine, where the reduction of ceramide release resulting from carnitine addition stimulated levels of insulin-like growth factor-1 (Di Marzio *et al*, 1999). Insulin growth factor-1 inhibits the dimerization of the apoptosis regulating proteins BCL-2-BAX in the mitochondrial membrane and also inhibits transcription from the BCL-2 promoter (Wang *et al*, 1998; Pugazenthi *et al*, 1999). Carnitine supplemented in combination with lipoic acid has also been indicated to prevent mitochondrial loss of cytochrome c, and thereby activation of caspase-3, in skeletal muscle of aging rats. This effect is speculated to be resulting from the protection of mitochondrial membrane integrity (Tamilselvan *et al*, 2007). Supplementation of acetylcarnitine in thioredoxin deficient cells prevents the induction of signaling events leading to apoptosis by suppressing oxidative stress in and around mitochondria (Zhu *et al*, 2007).

Recently, interesting claims have been made which describe a dual role for carnitine in regulating the onset of apoptosis. A number of studies report the addition of carnitine to have different effects on the regulation of apoptosis in cancer cells compared to normal cells. Observations in cancer cells such as HT-29 and U937 leukemic cells have suggested that apoptosis is increased by the addition of carnitine and carnitine derivatives (Wenzel *et al*, 2005; Ferrara *et al*, 2005). A recent study has indicated carnitine to induce apoptosis in mouse cancer cells, by inducing both the mitochondrial and Fas regulated signaling cascades (Fan *et al*, 2009). It is currently not clear by what mode of action carnitine addition would result in the divergent regulation in cancer cells compared to normal cell systems.

2.4.5. OXIDATIVE STRESS PROTECTION IN *S. CEREVISIAE*

Carnitine supplementation has recently been shown to have a stress protective effect in *S. cerevisiae* (Franken *et al*, 2008). This was shown to be specific for stress conditions induced by hydrogen peroxide and certain organic acids and independent of the carnitine shuttle. No protection by supplemented carnitine was observed for osmotic and thermal stresses. Interestingly, a role for *CAT2*, independent of carnitine seems evident in the protection against oxidative stress.

The work done in Chapter 5 points towards carnitine's effect against oxidative stress being specific and not due to an enhancement of the general response against oxidative stress. Intriguingly, while carnitine protects against certain ROS generating oxidants, an inverse and detrimental effect is observed in combination with thiol modifying agents. It is tempting to speculate a similar function involved in this context to be responsible for the diverse effects observed in the regulation of programmed cell death. Of further interest is the requirement of the cytochrome c heme lyase, Cyc3p, both carnitine related protection and toxicity. *CYC3* was also shown to be upregulated by the presence of carnitine during oxidative stress. This suggests that carnitine is not only able to maintain mitochondrial integrity by the action of the shuttle but possibly also by upregulation of cytochrome c content. This could possibly have an impact on both oxidative phosphorylation function as well as one of the main events associated with apoptosis induction, the release of cytochrome from the mitochondria.

2.5. CONCLUSION

Due to the central and role that carnitine and the carnitine shuttle play in eukaryotic energy metabolism, the composition and function of this system has been a topic of intensive research interest for the past 60 years. Understanding of the proteins and enzymes involved in this area of metabolism has greatly advanced knowledge of the far reaching impact that the carnitine shuttle has on the regulation of metabolism. As a consequence of this regulatory function, specifically on the concentrations of and ratios between compartmentalized pools of free CoA and acylated CoA, carnitine and its esters have a considerable potential as therapeutic agents or supplements in a diverse range of metabolic diseases. The modes of action of these molecules, mediated by the action of the shuttle enzymes are slowly emerging. In addition, it is becoming clear that not all phenotypic effects of carnitine administration can be attributed to its role in the

shuttling of intermediaries. The broadening of this knowledge base is comparatively slow, since most studies in this field are either clinical or diagnostic in nature and do not contribute to the understanding of the mechanisms underlying the effects of carnitine. In addition, carnitine and acylcarnitines appear to have distinct, but overlapping functions depending on the context of the cell or tissue system that is being studied, making it difficult to draw conclusions between systems.

In the yeast, *S. cerevisiae*, the carnitine shuttle shares its central metabolic function with that of higher eukaryotes. The carnitine acetyl-transferases and carnitine/acetylcarnitine transporter involved have been identified and localized within the cell. The functional and metabolic impact of these enzymes is, however, not as clearly understood as that of mammalian systems. Nevertheless, *S. cerevisiae* does present an ideal model system for the studies of carnitine and carnitine shuttle related phenotypes, since blockage of the glyoxylate bypass creates a system in which the shuttle and its effects can be studied using the wide array of genetic tools available in *S. cerevisiae*. Such studies benefit from the relative simplicity of the yeast metabolism, which is not under the same multidimensional control effective in a multicelullar system. Furthermore, *S. cerevisiae* is unable to synthesize its own carnitine, enabling precise control of the concentrations of carnitine in experimental conditions. Yeast is currently being advocated as an effective model for the study of the molecular effects associated with various metabolic and neurodegenerative diseases. The use of this system could therefore add greatly to our understanding of the function of carnitine within the context of the eukaryotic cell.

2.6. REFERENCES

Abdul, H. M., Calabrese, V., Calvani, M. and Butterfield, D. A. (2006). Acetyl-L-carnitine-induced up-regulation of heat shock proteins protects cortical neurons against amyloid-beta peptide 1-42-mediated oxidative stress and neurotoxicity: implications for Alzheimer's disease. *J Neurosci Res* **84,** 398-408.

Andre, B. (1995). An overview of membrane transport proteins in *Saccharomyces cerevisiae*. *Yeast* **11,** 1575-611.

Arduini, A., Bonomini, M., Savica, V., Amato, A. and Zammit, V. (2008). Carnitine in metabolic disease: potential for pharmacological intervention. *Pharmacol Ther* **120,** 149-56.

Arrigoni-Martelli, E. and Caso, V. (2001). Carnitine protects mitochondria and removes toxic acyls from xenobiotics. *Drugs Exp Clin Res* **27,** 27-49.

Bartlett, K. and Eaton, S. (2004). Mitochondrial beta-oxidation. *Eur J Biochem* **271,** 462-9.

Bieber, L. L. (1988). Carnitine. *Annu Rev Biochem* **57,** 261-83.

Biolo, G., Stulle, M., Bianco, F., Mengozzi, G., Barazzoni, R., Vasile, A., Panzetta, G. and Guarnieri, G. (2008). Insulin action on glucose and protein metabolism during L-carnitine supplementation in maintenance haemodialysis patients. *Nephrol Dial Transplant* **23,** 991-7.

Borum, P. R. and Broquist, H. P. (1977). Purification of S-adenosylmethionine: epsilon-N-L-lysine methyltransferase. The first enzyme in carnitine biosynthesis. *J Biol Chem* **252,** 5651-5.

Brady, L. J., Knoeber, C. M., Hoppel, C. L., Leathers, C. W., McFarland, D. and Brady, P. S. (1986). Pharmacologic action of L-carnitine on hypertriglyceridemia in obese Zucker rats. *Metabolism* **35,** 555-62.

Brass, E. P. (1994). Overview of coenzyme A metabolism and its role in cellular toxicity. *Chem Biol Interact* **90,** 203-14.

Brass, E. P., Adler, S., Sietsema, K. E., Hiatt, W. R., Orlando, A. M. and Amato, A. (2001). Intravenous L-carnitine increases plasma carnitine, reduces fatigue, and may preserve exercise capacity in hemodialysis patients. *Am J Kidney Dis* **37,** 1018-28.

Bremer, J. (1983). Carnitine: metabolism and functions. *Physiol Rev* **63,** 1420-80.

Broderick, T. L., Quinney, H. A. and Lopaschuk, G. D. (1992). Carnitine stimulation of glucose oxidation in the fatty acid perfused isolated working rat heart. *J Biol Chem* **267,** 3758-63.

Brown, N. F., Esser, V., Foster, D. W. and McGarry, J. D. (1994). Expression of a cDNA for rat liver carnitine palmitoyltransferase I in yeast establishes that catalytic activity and malonyl-CoA sensitivity reside in a single polypeptide. *J Biol Chem* **269,** 26438-42.

Brown, N. F., Sen, A., Soltis, D. A., Jones, B., Foster, D. W. and McGarry, J. D. (1993). Expression of precursor and mature carnitine palmitoyltransferase II in *Escherichia coli* and in vitro: differential behaviour of rat and human isoforms. *Biochem J* **294 (Pt 1),** 79-86.

Calabrese, V., Colombrita, C., Sultana, R., Scapagnini, G., Calvani, M., Butterfield, D. A. and Stella, A. M. (2006a). Redox modulation of heat shock protein expression by acetylcarnitine in aging brain: relationship to antioxidant status and mitochondrial function. *Antioxid Redox Signal* **8,** 404-16.

Calabrese, V., Giuffrida Stella, A. M., Calvani, M. and Butterfield, D. A. (2006b). Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes. *J Nutr Biochem* **17,** 73-88.

Calabrese, V., Ravagna, A., Colombrita, C., Scapagnini, G., Guagliano, E., Calvani, M., Butterfield, D. A. and Giuffrida Stella, A. M. (2005). Acetylcarnitine induces heme oxygenase in rat astrocytes and protects against oxidative stress: involvement of the transcription factor Nrf2. *J Neurosci Res* **79,** 509-21.

Cederblad, G. (1987). Effect of diet on plasma carnitine levels and urinary carnitine excretion in humans. *Am J Clin Nutr* **45,** 725-9.

Cederblad, G. and Lindstedt, S. (1972). A method for the determination of carnitine in the picomole range. *Clin Chim Acta* **37,** 235-43.

Chern, M. K. and Pietruszko, R. (1995). Human aldehyde dehydrogenase E3 isozyme is a betaine aldehyde dehydrogenase. *Biochem Biophys Res Commun* **213,** 561-8.

Coleman, R. A. and Lee, D. P. (2004). Enzymes of triacylglycerol synthesis and their regulation. *Prog Lipid Res* **43,** 134-76.

Corti, O., DiDonato, S. and Finocchiaro, G. (1994). Divergent sequences in the 5' region of cDNA suggest alternative splicing as a mechanism for the generation of carnitine acetyltransferases with

different subcellular localizations. *Biochem J* **303 (Pt 1),** 37-41.

Dai, T., Abou-Rjaily, G. A., Al-Share, Q. Y., Yang, Y., Fernstrom, M. A., Deangelis, A. M., Lee, A. D., Sweetman, L., Amato, A., Pasquali, M., Lopaschuk, G. D., Erickson, S. K. and Najjar, S. M. (2004). Interaction between altered insulin and lipid metabolism in CEACAM1-inactive transgenic mice. *J Biol Chem* **279,** 45155-61.

Dennis, S. C., Gevers, W. and Opie, L. H. (1991). Protons in ischemia: where do they come from; where do they go to? *J Mol Cell Cardiol* **23,** 1077-86.

Deufel, T. (1990). Determination of L-carnitine in biological fluids and tissues. *J Clin Chem Clin Biochem* **28,** 307-11.

Di Marzio, L., Alesse, E., Roncaioli, P., Muzi, P., Moretti, S., Marcellini, S., Amicosante, G., De Simone, C. and Cifone, M. G. (1997). Influence of L-carnitine on CD95 cross-lining-induced apoptosis and ceramide generation in human cell lines: correlation with its effects on purified acidic and neutral sphingomyelinases in vitro. *Proc Assoc Am Physicians* **109,** 154-63.

Di Marzio, L., Moretti, S., D'Alo, S., Zazzeroni, F., Marcellini, S., Smacchia, C., Alesse, E., Cifone, M. G. and De Simone, C. (1999). Acetyl-L-carnitine administration increases insulin-like growth factor 1 levels in asymptomatic HIV-1-infected subjects: correlation with its suppressive effect on lymphocyte apoptosis and ceramide generation. *Clin Immunol* **92,** 103-10.

Distler, A. M., Kerner, J. and Hoppel, C. L. (2007). Post-translational modifications of rat liver mitochondrial outer membrane proteins identified by mass spectrometry. *Biochim Biophys Acta* **1774,** 628-36.

Dunn, W. A., Rettura, G., Seifter, E. and Englard, S. (1984). Carnitine biosynthesis from gamma-butyrobetaine and from exogenous protein-bound 6-N-trimethyl-L-lysine by the perfused guinea pig liver. Effect of ascorbate deficiency on the in situ activity of gamma-butyrobetaine hydroxylase. *J Biol Chem* **259,** 10764-70.

Eaton, S. and Bartlett, K. (1996). Inhibition of mitochondrial beta-oxidation in the heart by increased redox state of the ubiquinone pool. *Biochem Soc Trans* **24,** 457S.

Eaton, S., Fukumoto, K., Stefanutti, G., Spitz, L., Zammit, V. A. and Pierro, A. (2003). Myocardial carnitine palmitoyltransferase I as a target for oxidative modification in inflammation and sepsis. *Biochem Soc Trans* **31,** 1133-6.

Elgersma, Y., van Roermund, C. W., Wanders, R. J. and Tabak, H. F. (1995). Peroxisomal and mitochondrial carnitine acetyltransferases of *Saccharomyces cerevisiae* are encoded by a single gene. *Embo J* **14,** 3472-9.

Enomoto, A., Wempe, M. F., Tsuchida, H., Shin, H. J., Cha, S. H., Anzai, N., Goto, A., Sakamoto, A., Niwa, T., Kanai, Y., Anders, M. W. and Endou, H. (2002). Molecular identification of a novel carnitine transporter specific to human testis. Insights into the mechanism of carnitine recognition. *J Biol Chem* **277,** 36262-71.

Evans, A. M. and Fornasini, G. (2003). Pharmacokinetics of L-carnitine. *Clin Pharmacokinet* **42,** 941-67.

Fan, J. P., Kim, H. S. and Han, G. D. (2009). Induction of apoptosis by L-carnitine through regulation of two main pathways in Hepa1c1c 7 cells. *Amino Acids* **36,** 365-72.

Felix, C., Gillis, M., Driedzic, W. R., Paulson, D. J. and Broderick, T. L. (2001). Effects of propionyl-L-carnitine on isolated mitochondrial function in the reperfused diabetic rat heart. *Diabetes Res Clin Pract* **53,** 17-24.

Ferrara, F., Bertelli, A. and Falchi, M. (2005). Evaluation of carnitine, acetylcarnitine and isovalerylcarnitine on immune function and apoptosis. *Drugs Exp Clin Res* **31,** 109-14.

Fingerhut, R., Roschinger, W., Muntau, A. C., Dame, T., Kreischer, J., Arnecke, R., Superti-Furga, A., Troxler, H., Liebl, B., Olgemoller, B. and Roscher, A. A. (2001). Hepatic carnitine palmitoyltransferase I deficiency: acylcarnitine profiles in blood spots are highly specific. *Clin Chem* **47,** 1763-8.

Fontaine, M., Briand, G., Ser, N., Armelin, I., Rolland, M. O., Degand, P. and Vamecq, J. (1996). Metabolic studies in twin brothers with 2-methylacetoacetyl-CoA thiolase deficiency. *Clin Chim Acta* **255,** 67-83.

Foster, D. W. (2004). The role of the carnitine system in human metabolism. *Ann N Y Acad Sci* **1033,** 1-16.

Fraenkel, G. (1954). The distribution of vitamin BT (carnitine) throughout the animal kingdom. *Arch Biochem Biophys* **50,** 486-95.

Franken, J., Kroppenstedt, S., Swiegers, J. H. and Bauer, F. F. (2008). Carnitine and carnitine acetyltransferases in the yeast *Saccharomyces cerevisiae*: a role for carnitine in stress protection. *Curr Genet* **53,** 347-60.

Fukumoto, K., Pierro, A., Spitz, L. and Eaton, S. (2002). Differential effects of neonatal endotoxemia on heart and kidney carnitine palmitoyl transferase I. *J Pediatr Surg* **37,** 723-6.

Fukumoto, K., Pierro, A., Zammit, V. A., Spitz, L. and Eaton, S. (2004). Tyrosine nitration of carnitine palmitoyl transferase I during endotoxaemia in suckling rats. *Biochim Biophys Acta* **1683,** 1-6.

Ganapathy, M. E., Huang, W., Rajan, D. P., Carter, A. L., Sugawara, M., Iseki, K., Leibach, F. H. and Ganapathy, V. (2000). beta-lactam antibiotics as substrates for OCTN2, an organic cation/carnitine transporter. *J Biol Chem* **275,** 1699-707.

Gempel, K., Kiechl, S., Hofmann, S., Lochmuller, H., Kiechl-Kohlendorfer, U., Willeit, J., Sperl, W., Rettinger, A., Bieger, I., Pongratz, D., Gerbitz, K. D. and Bauer, M. F. (2002). Screening for carnitine palmitoyltransferase II deficiency by tandem mass spectrometry. *J Inherit Metab Dis* **25,** 17-27.

Gulcin, I. (2006). Antioxidant and antiradical activities of L-carnitine. *Life Sci* **78,** 803-11.

Gunal, A. I., Celiker, H., Donder, E. and Gunal, S. Y. (1999). The effect of L-carnitine on insulin resistance in hemodialysed patients with chronic renal failure. *J Nephrol* **12,** 38-40.

Hagen, T. M., Ingersoll, R. T., Wehr, C. M., Lykkesfeldt, J., Vinarsky, V., Bartholomew, J. C., Song, M. H. and Ames, B. N. (1998). Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. *Proc Natl Acad Sci U S A* **95,** 9562-6.

Henderson, L. M., Nelson, P. J. and Henderson, L. (1982). Mammalian enzymes of trimethyllysine conversion to trimethylaminobutyrate. *Fed Proc* **41,** 2843-7.

Hulse, J. D., Ellis, S. R. and Henderson, L. M. (1978). Carnitine biosynthesis. beta-Hydroxylation of trimethyllysine by an alpha-ketoglutarate-dependent mitochondrial dioxygenase. *J Biol Chem* **253,** 1654-9.

Kahonen, M. T. (1979). Clofibrate-induced increase in carnitine and acylcarnitine content in rats. *Biochem Pharmacol* **28,** 3674-7.

Kelley, D. E. and Mandarino, L. J. (2000). Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* **49,** 677-83.

Kerner, J., Distler, A. M., Minkler, P., Parland, W., Peterman, S. M. and Hoppel, C. L. (2004). Phosphorylation of rat liver mitochondrial carnitine palmitoyltransferase-I: effect on the kinetic properties of the enzyme. *J Biol Chem* **279,** 41104-13.

Kerner, J. and Hoppel, C. (1998). Genetic disorders of carnitine metabolism and their nutritional management. *Annu Rev Nutr* **18,** 179-206.

Khan-Siddiqui, L. and Bamji, M. S. (1980). Plasma carnitine levels in adult males in India: effects of high cereal, low fat diet, fat supplementation, and nutrition status. *Am J Clin Nutr* **33,** 1259-63.

Kikonyogo, A. and Pietruszko, R. (1996). Aldehyde dehydrogenase from adult human brain that dehydrogenates gamma-aminobutyraldehyde: purification, characterization, cloning and distribution. *Biochem J* **316 (Pt 1),** 317-24.

Kispal, G., Sumegi, B., Dietmeier, K., Bock, I., Gajdos, G., Tomcsanyi, T. and Sandor, A. (1993). Cloning and sequencing of a cDNA encoding *Saccharomyces cerevisiae* carnitine acetyltransferase. Use of the cDNA in gene disruption studies. *J Biol Chem* **268,** 1824-9.

Koizumi, A., Nozaki, J., Ohura, T., Kayo, T., Wada, Y., Nezu, J., Ohashi, R., Tamai, I., Shoji, Y., Takada, G., Kibira, S., Matsuishi, T. and Tsuji, A. (1999). Genetic epidemiology of the carnitine transporter OCTN2 gene in a Japanese population and phenotypic characterization in Japanese pedigrees with primary systemic carnitine deficiency. *Hum Mol Genet* **8,** 2247-54.

Kopec, B. and Fritz, I. B. (1973). Comparison of properties of carnitine palmitoyltransferase I with those of carnitine palmitoyltransferase II, and preparation of antibodies to carnitine palmitoyltransferases. *J Biol Chem* **248,** 4069-74.

Kunau, W. H., Dommes, V. and Schulz, H. (1995). beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. *Prog Lipid Res* **34,** 267-342.

Kurys, G., Shah, P. C., Kikonygo, A., Reed, D., Ambroziak, W. and Pietruszko, R. (1993). Human aldehyde dehydrogenase. cDNA cloning and primary structure of the enzyme that catalyzes dehydrogenation of 4-aminobutyraldehyde. *Eur J Biochem* **218,** 311-20.

Kwan, B. C., Kronenberg, F., Beddhu, S. and Cheung, A. K. (2007). Lipoprotein metabolism and lipid management in chronic kidney disease. *J Am Soc Nephrol* **18,** 1246-61.

LaBadie, J., Dunn, W. A. and Aronson, N. N., Jr. (1976). Hepatic synthesis of carnitine from protein-bound trimethyl-lysine. Lysosomal digestion of methyl-lysine-labelled asialo-fetuin. *Biochem J* **160,** 85-95.

Lahjouji, K., Mitchell, G. A. and Qureshi, I. A. (2001). Carnitine transport by organic cation transporters and systemic carnitine deficiency. *Mol Genet Metab* **73,** 287-97.

Lee, J., Lee, B., Shin, D., Kwak, S. S., Bahk, J. D., Lim, C. O. and Yun, D. J. (2002). Carnitine uptake by *AGP2* in yeast *Saccharomyces cerevisiae* is dependent on Hog1 MAP kinase pathway. *Mol Cells* **13,** 407-12.

Leenders, F., Tesdorpf, J. G., Markus, M., Engel, T., Seedorf, U. and Adamski, J. (1996). Porcine 80-kDa protein reveals intrinsic 17 beta-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer activities. *J Biol Chem* **271,** 5438-42.

Lennon, D. L., Shrago, E. R., Madden, M., Nagle, F. J. and Hanson, P. (1986). Dietary carnitine intake related to skeletal muscle and plasma carnitine concentrations in adult men and women. *Am J Clin Nutr* **43,** 234-8.

Lin, S. W., Chen, J. C., Hsu, L. C., Hsieh, C. L. and Yoshida, A. (1996). Human gamma-

aminobutyraldehyde dehydrogenase (ALDH9): cDNA sequence, genomic organization, polymorphism, chromosomal localization, and tissue expression. *Genomics* **34,** 376-80.

Liu, J., Killilea, D. W. and Ames, B. N. (2002). Age-associated mitochondrial oxidative decay: improvement of carnitine acetyltransferase substrate-binding affinity and activity in brain by feeding old rats acetyl-L- carnitine and/or R-alpha -lipoic acid. *Proc Natl Acad Sci U S A* **99,** 1876-81.

Liu, J. and Rosner, M. H. (2006). Lipid abnormalities associated with end-stage renal disease. *Semin Dial* **19,** 32-40.

Lombard, K. A., Olson, A. L., Nelson, S. E. and Rebouche, C. J. (1989). Carnitine status of lactoovovegetarians and strict vegetarian adults and children. *Am J Clin Nutr* **50,** 301-6.

Lopaschuk, G. D. (2004). Targets for modulation of fatty acid oxidation in the heart. *Curr Opin Investig Drugs* **5,** 290-4.

Lopaschuk, G. D., Rebeyka, I. M. and Allard, M. F. (2002). Metabolic modulation: a means to mend a broken heart. *Circulation* **105,** 140-2.

Lorenz, M. C., Bender, J. A. and Fink, G. R. (2004). Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3,** 1076-87.

Loster, H., Keller, T., Grommisch, J. and Grunder, W. (1999). Effects of L-carnitine and its acetyl and propionyl esters on ATP and PCr levels of isolated rat hearts perfused without fatty acids and investigated by means of 31P-NMR spectroscopy. *Mol Cell Biochem* **200,** 93-102.

Markwell, M. A., McGroarty, E. J., Bieber, L. L. and Tolbert, N. E. (1973). The subcellular distribution of carnitine acyltransferases in mammalian liver and kidney. A new peroxisomal enzyme. *J Biol Chem* **248,** 3426-32.

Marsh, J. B., Welty, F. K. and Schaefer, E. J. (2000). Stable isotope turnover of apolipoproteins of high-density lipoproteins in humans. *Curr Opin Lipidol* **11,** 261-6.

McDaniel, M. A., Maier, S. F. and Einstein, G. O. (2003). "Brain-specific" nutrients: a memory cure? *Nutrition* **19,** 957-75.

McGarry, J. D. and Brown, N. F. (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem* **244,** 1-14.

McGarry, J. D. and Brown, N. F. (2000). Reconstitution of purified, active and malonyl-CoA-sensitive rat liver carnitine palmitoyltransferase I: relationship between membrane environment and malonyl-CoA sensitivity. *Biochem J* **349,** 179-87.

Moder, M., Kiessling, A., Loster, H. and Bruggemann, L. (2003). The pattern of urinary acylcarnitines determined by electrospray mass spectrometry: a new tool in the diagnosis of diabetes mellitus. *Anal Bioanal Chem* **375,** 200-10.

Moretti, S., Alesse, E., Di Marzio, L., Zazzeroni, F., Ruggeri, B., Marcellini, S., Famularo, G., Steinberg, S. M., Boschini, A., Cifone, M. G. and De Simone, C. (1998). Effect of L-carnitine on human immunodeficiency virus-1 infection-associated apoptosis: a pilot study. *Blood* **91,** 3817-24.

Muller, D. M., Seim, H., Kiess, W., Loster, H. and Richter, T. (2002). Effects of oral L-carnitine supplementation on in vivo long-chain fatty acid oxidation in healthy adults. *Metabolism* **51,** 1389-91.

Murthy, M. S. and Pande, S. V. (1985). Microcompartmentation of transported carnitine, acetylcarnitine and ADP occurs in the mitochondrial matrix. Implications for transport measurements and

metabolism. *Biochem J* **230,** 657-63.

Mutomba, M. C., Yuan, H., Konyavko, M., Adachi, S., Yokoyama, C. B., Esser, V., McGarry, J. D., Babior, B. M. and Gottlieb, R. A. (2000). Regulation of the activity of caspases by L-carnitine and palmitoylcarnitine. *FEBS Lett* **478,** 19-25.

Nakanishi, T., Hatanaka, T., Huang, W., Prasad, P. D., Leibach, F. H., Ganapathy, M. E. and Ganapathy, V. (2001). Na+- and Cl--coupled active transport of carnitine by the amino acid transporter ATB(0,+) from mouse colon expressed in HRPE cells and Xenopus oocytes. *J Physiol* **532,** 297-304.

Palmieri, L., Lasorsa, F. M., Iacobazzi, V., Runswick, M. J., Palmieri, F. and Walker, J. E. (1999). Identification of the mitochondrial carnitine carrier in *Saccharomyces cerevisiae*. *FEBS Lett* **462,** 472-6.

Panter, R. A. and Mudd, J. B. (1969). Carnitine levels in some higher plants. *FEBS Lett* **5,** 169-170.

Parnetti, L., Gaiti, A., Mecocci, P., Cadini, D. and Senin, U. (1992). Pharmacokinetics of IV and oral acetyl-L-carnitine in a multiple dose regimen in patients with senile dementia of Alzheimer type. *Eur J Clin Pharmacol* **42,** 89-93.

Pastorino, J. G., Snyder, J. W., Serroni, A., Hoek, J. B. and Farber, J. L. (1993). Cyclosporin and carnitine prevent the anoxic death of cultured hepatocytes by inhibiting the mitochondrial permeability transition. *J Biol Chem* **268,** 13791-8.

Petersen, K. F., Dufour, S., Befroy, D., Lehrke, M., Hendler, R. E. and Shulman, G. I. (2005). Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* **54,** 603-8.

Poirier, M., Vincent, G., Reszko, A. E., Bouchard, B., Kelleher, J. K., Brunengraber, H. and Des Rosiers, C. (2002). Probing the link between citrate and malonyl-CoA in perfused rat hearts. *Am J Physiol Heart Circ Physiol* **283,** H1379-86.

Power, R. A., Hulver, M. W., Zhang, J. Y., Dubois, J., Marchand, R. M., Ilkayeva, O., Muoio, D. M. and Mynatt, R. L. (2007). Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia* **50,** 824-32.

Price, N., van der Leij, F., Jackson, V., Corstorphine, C., Thomson, R., Sorensen, A. and Zammit, V. (2002). A novel brain-expressed protein related to carnitine palmitoyltransferase I. *Genomics* **80,** 433-42.

Prichard, S. S. (2003). Impact of dyslipidemia in end-stage renal disease. *J Am Soc Nephrol* **14,** S315-20.

Prigneau, O., Porta, A. and Maresca, B. (2004). *Candida albicans* CTN gene family is induced during macrophage infection: homology, disruption and phenotypic analysis of *CTN3* gene. *Fungal Genet Biol* **41,** 783-93.

Prip-Buus, C., Cohen, I., Kohl, C., Esser, V., McGarry, J. D. and Girard, J. (1998). Topological and functional analysis of the rat liver carnitine palmitoyltransferase 1 expressed in *Saccharomyces cerevisiae*. *FEBS Lett* **429,** 173-8.

Pugazhenthi, S., Miller, E., Sable, C., Young, P., Heidenreich, K. A., Boxer, L. M. and Reusch, J. E. (1999). Insulin-like growth factor-I induces bcl-2 promoter through the transcription factor cAMP-response element-binding protein. *J Biol Chem* **274,** 27529-35.

Rajasekar, P. and Anuradha, C. V. (2007). Effect of L-carnitine on skeletal muscle lipids and oxidative stress in rats fed high-fructose diet. *Exp Diabetes Res* **2007,** 72741.

Ramsay, R. R. (1999). The role of the carnitine system in peroxisomal fatty acid oxidation. *Am J Med Sci* **318,** 28-35.

Ramsay, R. R. and Arduini, A. (1993). The carnitine acyltransferases and their role in modulating acyl-CoA pools. *Arch Biochem Biophys* **302,** 307-14.

Ramsay, R. R., Gandour, R. D. and van der Leij, F. R. (2001). Molecular enzymology of carnitine transfer and transport. *Biochim Biophys Acta* **1546,** 21-43.

Ramsay, R. R. and Naismith, J. H. (2003). A snapshot of carnitine acetyltransferase. *Trends Biochem Sci* **28,** 343-6.

Ramsay, R. R. and Zammit, V. A. (2004). Carnitine acyltransferases and their influence on CoA pools in health and disease. *Mol Aspects Med* **25,** 475-93.

Rebouche, C. J. and Engel, A. G. (1984). Kinetic compartmental analysis of carnitine metabolism in the human carnitine deficiency syndromes. Evidence for alterations in tissue carnitine transport. *J Clin Invest* **73,** 857-67.

Reddy, J. K. and Mannaerts, G. P. (1994). Peroxisomal lipid metabolism. *Annu Rev Nutr* **14,** 343-70.

Reszko, A. E., Kasumov, T., David, F., Jobbins, K. A., Thomas, K. R., Hoppel, C. L., Brunengraber, H. and Des Rosiers, C. (2004). Peroxisomal fatty acid oxidation is a substantial source of the acetyl moiety of malonyl-CoA in rat heart. *J Biol Chem* **279,** 19574-9.

Rodrigues, B., Xiang, H. and McNeill, J. H. (1988). Effect of L-carnitine treatment on lipid metabolism and cardiac performance in chronically diabetic rats. *Diabetes* **37,** 1358-64.

Roomets, E., Kivela, T. and Tyni, T. (2008). Carnitine palmitoyltransferase I and Acyl-CoA dehydrogenase 9 in retina: insights of retinopathy in mitochondrial trifunctional protein defects. *Invest Ophthalmol Vis Sci* **49,** 1660-4.

Rudman, D., Sewell, C. W. and Ansley, J. D. (1977). Deficiency of carnitine in cachectic cirrhotic patients. *J Clin Invest* **60,** 716-23.

Rufer, A. C., Thoma, R. and Hennig, M. (2009). Structural insight into function and regulation of carnitine palmitoyltransferase. *Cell Mol Life Sci*.

Sachan, D. S. and Broquist, H. P. (1980). Synthesis of carnitine from epsilon-N-trimethyllysine in post mitochondrial fractions of *Neurospora crassa*. *Biochem Biophys Res Commun* **96,** 870-5.

Sachan, D. S. and Hoppel, C. L. (1980). Carnitine biosynthesis. Hydroxylation of N6-trimethyl-lysine to 3-hydroxy-N6-trimethyl-lysine. *Biochem J* **188,** 529-34.

Saddik, M., Gamble, J., Witters, L. A. and Lopaschuk, G. D. (1993). Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart. *J Biol Chem* **268,** 25836-45.

Sandor, A., Kispal, G., Melegh, B. and Alkonyi, I. (1985). Release of carnitine from the perfused rat liver. *Biochim Biophys Acta* **835,** 83-91.

Scaglia, F., Wang, Y. and Longo, N. (1999). Functional characterization of the carnitine transporter defective in primary carnitine deficiency. *Arch Biochem Biophys* **364,** 99-106.

Scaglia, F., Wang, Y., Singh, R. H., Dembure, P. P., Pasquali, M., Fernhoff, P. M. and Longo, N. (1998). Defective urinary carnitine transport in heterozygotes for primary carnitine deficiency. *Genet Med* **1,** 34-9.

Schmalix, W. and Bandlow, W. (1993). The ethanol-inducible *YAT1* gene from yeast encodes a presumptive mitochondrial outer carnitine acetyltransferase. *J Biol Chem* **268,** 27428-39.

Schmitz-Peiffer, C. (2000). Signalling aspects of insulin resistance in skeletal muscle: mechanisms

induced by lipid oversupply. *Cell Signal* **12,** 583-94.

Schonekess, B. O., Allard, M. F. and Lopaschuk, G. D. (1995). Propionyl L-carnitine improvement of hypertrophied rat heart function is associated with an increase in cardiac efficiency. *Eur J Pharmacol* **286,** 155-66.

Schulz, H. (1991). Beta oxidation of fatty acids. *Biochim Biophys Acta* **1081,** 109-20.

Shi, J., Zhu, H., Arvidson, D. N., Cregg, J. M. and Woldegiorgis, G. (1998). Deletion of the conserved first 18 N-terminal amino acid residues in rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA sensitivity and binding. *Biochemistry* **37,** 11033-8.

Shi, J., Zhu, H., Arvidson, D. N. and Woldegiorgis, G. (1999). A single amino acid change (substitution of glutamate 3 with alanine) in the N-terminal region of rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA inhibition and high affinity binding. *J Biol Chem* **274,** 9421-6.

Sierra, A. Y., Gratacos, E., Carrasco, P., Clotet, J., Urena, J., Serra, D., Asins, G., Hegardt, F. G. and Casals, N. (2008). CPT1c is localized in endoplasmic reticulum of neurons and has carnitine palmitoyltransferase activity. *J Biol Chem* **283,** 6878-85.

Silva-Adaya, D., Perez-De La Cruz, V., Herrera-Mundo, M. N., Mendoza-Macedo, K., Villeda-Hernandez, J., Binienda, Z., Ali, S. F. and Santamaria, A. (2008). Excitotoxic damage, disrupted energy metabolism, and oxidative stress in the rat brain: antioxidant and neuroprotective effects of L-carnitine. *J Neurochem* **105,** 677-89.

Sim, K. G., Wiley, V., Carpenter, K. and Wilcken, B. (2001). Carnitine palmitoyltransferase I deficiency in neonate identified by dried blood spot free carnitine and acylcarnitine profile. *J Inherit Metab Dis* **24,** 51-9.

Sloan, J. L. and Mager, S. (1999). Cloning and functional expression of a human Na(+) and Cl(-)-dependent neutral and cationic amino acid transporter B(0+). *J Biol Chem* **274,** 23740-5.

Spiekerkoetter, U., Tokunaga, C., Wendel, U., Mayatepek, E., Exil, V., Duran, M., Wijburg, F. A., Wanders, R. J. and Strauss, A. W. (2004). Changes in blood carnitine and acylcarnitine profiles of very long-chain acyl-CoA dehydrogenase-deficient mice subjected to stress. *Eur J Clin Invest* **34,** 191-6.

Stanley, C. A. (2004). Carnitine deficiency disorders in children. *Ann N Y Acad Sci* **1033,** 42-51.

Stanley, W. C. (2005). Diabetes and ischaemic heart disease: essential role for metabolic therapies. *Coron Artery Dis* **16 Suppl 1,** S1.

Steiber, A., Kerner, J. and Hoppel, C. L. (2004). Carnitine: a nutritional, biosynthetic, and functional perspective. *Mol Aspects Med* **25,** 455-73.

Stein, R. and Englard, S. (1981). The use of a tritium release assay to measure 6-N-trimethyl-L-lysine hydroxylase activity: synthesis of 6-N-[3-3H]trimethyl-DL-lysine. *Anal Biochem* **116,** 230-6.

Stemple, C. J., Davis, M. A. and Hynes, M. J. (1998). The facC gene of *Aspergillus nidulans* encodes an acetate-inducible carnitine acetyltransferase. *J Bacteriol* **180,** 6242-51.

Stephens, F. B., Constantin-Teodosiu, D. and Greenhaff, P. L. (2007). New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *J Physiol* **581,** 431-44.

Stephens, F. B., Constantin-Teodosiu, D., Laithwaite, D., Simpson, E. J. and Greenhaff, P. L. (2006). An acute increase in skeletal muscle carnitine content alters fuel metabolism in resting human skeletal muscle. *J Clin Endocrinol Metab* **91,** 5013-8.

Strijbis, K., van Roermund, C. W., Hardy, G. P., van den Burg, J., Bloem, K., de Haan, J., van Vlies, N.,

Wanders, R. J., Vaz, F. M. and Distel, B. (2009). Identification and characterization of a complete carnitine biosynthesis pathway in *Candida albicans*. *Faseb J*.

Strijbis, K., van Roermund, C. W., Visser, W. F., Mol, E. C., van den Burg, J., MacCallum, D. M., Odds, F. C., Paramonova, E., Krom, B. P. and Distel, B. (2008). Carnitine-dependent transport of acetyl coenzyme A in *Candida albicans* is essential for growth on nonfermentable carbon sources and contributes to biofilm formation. *Eukaryot Cell* **7,** 610-8.

Summers, S. A. (2006). Ceramides in insulin resistance and lipotoxicity. *Prog Lipid Res* **45,** 42-72.

Swiegers, J. H., Dippenaar, N., Pretorius, I. S. and Bauer, F. F. (2001). Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: three carnitine acetyltransferases are essential in a carnitine-dependent strain. *Yeast* **18,** 585-95.

Swiegers, J. H., Vaz, F. M., Pretorius, I. S., Wanders, R. J. and Bauer, F. F. (2002). Carnitine biosynthesis in *Neurospora crassa*: identification of a cDNA coding for epsilon-N-trimethyllysine hydroxylase and its functional expression in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **210,** 19-23.

Tamai, I., Ohashi, R., Nezu, J., Yabuuchi, H., Oku, A., Shimane, M., Sai, Y. and Tsuji, A. (1998). Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *J Biol Chem* **273,** 20378-82.

Tamai, I., Ohashi, R., Nezu, J. I., Sai, Y., Kobayashi, D., Oku, A., Shimane, M. and Tsuji, A. (2000). Molecular and functional characterization of organic cation/carnitine transporter family in mice. *J Biol Chem* **275,** 40064-72.

Tamai, I., Yabuuchi, H., Nezu, J., Sai, Y., Oku, A., Shimane, M. and Tsuji, A. (1997). Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. *FEBS Lett* **419,** 107-11.

Tamilselvan, J., Jayaraman, G., Sivarajan, K. and Panneerselvam, C. (2007). Age-dependent upregulation of p53 and cytochrome c release and susceptibility to apoptosis in skeletal muscle fiber of aged rats: role of carnitine and lipoic acid. *Free Radic Biol Med* **43,** 1656-69.

Tein, I. (2003). Carnitine transport: pathophysiology and metabolism of known molecular defects. *J Inherit Metab Dis* **26,** 147-69.

Vacha, G. M., Giorcelli, G., d'Iddio, S., Valentini, G., Bagiella, E., Procopio, A., di Donato, S., Ashbrook, D. and Corsi, M. (1989). L-carnitine addition to dialysis fluid. A therapeutic alternative for hemodialysis patients. *Nephron* **51,** 237-42.

Vacha, G. M., Giorcelli, G., Siliprandi, N. and Corsi, M. (1983). Favorable effects of L-carnitine treatment on hypertriglyceridemia in hemodialysis patients: decisive role of low levels of high-density lipoprotein-cholesterol. *Am J Clin Nutr* **38,** 532-40.

van der Leij, F. R., Huijkman, N. C., Boomsma, C., Kuipers, J. R. and Bartelds, B. (2000). Genomics of the human carnitine acyltransferase genes. *Mol Genet Metab* **71,** 139-53.

van Roermund, C. W., Elgersma, Y., Singh, N., Wanders, R. J. and Tabak, H. F. (1995). The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. *Embo J* **14,** 3480-6.

Van Roermund, C. W., Hettema, E. H., Van Den Berg, M., Tabak, H. F. and Wanders, R. J. (1999). Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine

transporter, Agp2p. *EMBO J* **18,** 5843-52.

Vaz, F. M., Fouchier, S. W., Ofman, R., Sommer, M. and Wanders, R. J. (2000). Molecular and biochemical characterization of rat gamma-trimethylaminobutyraldehyde dehydrogenase and evidence for the involvement of human aldehyde dehydrogenase 9 in carnitine biosynthesis. *J Biol Chem* **275,** 7390-4.

Vaz, F. M., van Gool, S., Ofman, R., Ijlst, L. and Wanders, R. J. (1998). Carnitine biosynthesis: identification of the cDNA encoding human gamma-butyrobetaine hydroxylase. *Biochem Biophys Res Commun* **250,** 506-10.

Vaz, F. M. and Wanders, R. J. (2002). Carnitine biosynthesis in mammals. *Biochem J* **361,** 417-29.

Vesela, E., Racek, J., Trefil, L., Jankovy'ch, V. and Pojer, M. (2001). Effect of L-carnitine supplementation in hemodialysis patients. *Nephron* **88,** 218-23.

Wanders, R. J., Denis, S., Ruiter, J. P., Schutgens, R. B., van Roermund, C. W. and Jacobs, B. S. (1995). Measurement of peroxisomal fatty acid beta-oxidation in cultured human skin fibroblasts. *J Inherit Metab Dis* **18 Suppl 1,** 113-24.

Wang, L., Ma, W., Markovich, R., Chen, J. W. and Wang, P. H. (1998). Regulation of cardiomyocyte apoptotic signaling by insulin-like growth factor I. *Circ Res* **83,** 516-22.

Wang, Y., Korman, S. H., Ye, J., Gargus, J. J., Gutman, A., Taroni, F., Garavaglia, B. and Longo, N. (2001). Phenotype and genotype variation in primary carnitine deficiency. *Genet Med* **3,** 387-92.

Wenzel, U., Nickel, A. and Daniel, H. (2005). Increased carnitine-dependent fatty acid uptake into mitochondria of human colon cancer cells induces apoptosis. *J Nutr* **135,** 1510-4.

West, D. W., Chase, J. F. and Tubbs, P. K. (1971). The separation and properties of two forms of carnitine palmitoyltransferase from ox liver mitochondria. *Biochem Biophys Res Commun* **42,** 912-8.

Wilcken, B., Wiley, V., Sim, K. G. and Carpenter, K. (2001). Carnitine transporter defect diagnosed by newborn screening with electrospray tandem mass spectrometry. *J Pediatr* **138,** 581-4.

Wiseman, L. R. and Brogden, R. N. (1998). Propionyl-L-carnitine. *Drugs Aging* **12,** 243-8; discussion 249-50.

Wu, X., George, R. L., Huang, W., Wang, H., Conway, S. J., Leibach, F. H. and Ganapathy, V. (2000). Structural and functional characteristics and tissue distribution pattern of rat OCTN1, an organic cation transporter, cloned from placenta. *Biochim Biophys Acta* **1466,** 315-27.

Wu, X., Huang, W., Prasad, P. D., Seth, P., Rajan, D. P., Leibach, F. H., Chen, J., Conway, S. J. and Ganapathy, V. (1999). Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. *J Pharmacol Exp Ther* **290,** 1482-92.

Wutzke, K. D. and Lorenz, H. (2004). The effect of l-carnitine on fat oxidation, protein turnover, and body composition in slightly overweight subjects. *Metabolism* **53,** 1002-6.

Zammit, V. A. (1999). Carnitine acyltransferases: functional significance of subcellular distribution and membrane topology. *Prog Lipid Res* **38,** 199-224.

Zammit, V. A. (2008). Carnitine palmitoyltransferase 1: central to cell function. *IUBMB Life* **60,** 347-54.

Zammit, V. A., Corstorphine, C. G., Kolodziej, M. P. and Fraser, F. (1998). Lipid molecular order in liver mitochondrial outer membranes, and sensitivity of carnitine palmitoyltransferase I to malonyl-CoA. *Lipids* **33,** 371-6.

Zhou, H. and Lorenz, M. C. (2008). Carnitine acetyltransferases are required for growth on non-fermentable carbon sources but not for pathogenesis in *Candida albicans*. *Microbiology* **154,** 500-9.

Zhu, X., Sato, E. F., Wang, Y., Nakamura, H., Yodoi, J. and Inoue, M. (2008). Acetyl-L-carnitine suppresses apoptosis of thioredoxin 2-deficient DT40 cells. *Arch Biochem Biophys* **478,** 154-60.

Chapter 3

RESEARCH RESULTS I

Carnitine and carnitine acetyltransferases in the yeast *Saccharomyces cerevisiae*: A role for carnitine in stress protection.

ABSTRACT

To date, the only reported metabolic and physiological roles for carnitine in *Saccharomyces cerevisiae* are related to the activity of the carnitine shuttle. In yeast, the shuttle transfers peroxisomal activated acetyl-residues to the mitochondria. However, acetyl-CoA can also be metabolised by the glyoxylate cycle to form succinate. The two pathways therefore provide a metabolic bypass for each other, and carnitine-dependent phenotypes have only been described in strains with non-functional peroxisomal citrate synthase, Cit2p. Here we present evidence for a role of carnitine in stress protection that is independent of *CIT2* and of the carnitine shuttle. The data show that carnitine improves growth during oxidative stress and in the presence of weak organic acids in WT and in CAT deletion strains. Our data also show that strains with single, double and triple deletions of the three CAT genes generally present identical phenotypes, but that the deletion of *CAT2* decreases survival during oxidative stress in a carnitine-independent manner. Overexpression of single CAT genes does not lead to cross-complementation, suggesting a highly specific metabolic role for each enzyme. The data suggest that carnitine protects cells from oxidative and organic acid stress, while *CAT2* contributes to the response to oxidative stress.

3.1. INTRODUCTION

The β-oxidation of fatty acids in mammalian cells takes place in both mitochondria and peroxisomes. Medium and long-chain fatty acids are catabolised primarily in mitochondria whereas very long-chain fatty acids and certain branched-chain fatty acids are broken down primarily by peroxisomes (Wanders *et al*. 1995; Leenders *et al*. 1996; Schulz 1991). It is generally accepted that mammalian fatty acid β-oxidation in peroxisomes is incomplete and only involves chain shortening of fatty acids to produce acetyl-CoA and/or propionyl-CoA plus medium-chain acyl-CoAs. These are then transported as carnitine esters to the mitochondria, where they are further oxidised to CO_2 and H_2O (Bieber 1988; Reddy and Mannaerts 1994). The importance of peroxisomal β-oxidation is emphasised by the existence of inherited diseases in man that are caused by an impairment in peroxisomal β-oxidation (Wanders *et al*. 1995).

In contrast, the degradation of fatty acids in yeast takes place exclusively in peroxisomes (Kunau *et al*. 1995). The acetyl-CoA produced in the peroxisome has to be transported to the mitochondria for complete oxidation to CO_2 and H_2O. Two pathways (Figure. 3.1) for further utilisation of peroxisomal acetyl-CoA have been identified (Van Roermund *et al*. 1995). In the first, acetyl-CoA enters the peroxisomal glyoxylate cycle, a net producer of succinate, which is subsequently transported to the mitochondria, probably via a putative dicarboxylate carrier, Acr1p (Palmieri *et al*. 1997). The second pathway involves the intraperoxisomal transfer of the activated acetyl from CoA to carnitine, which is catalysed by carnitine acetyltransferase (CAT). Acetylcarnitine is subsequently transported to the mitochondria through the carnitine acetylcarnitine translocase Crc1p (Palmieri *et al*. 1997; Van Roermund *et al*. 1995). Mitochondrial CAT catalyses the reverse reaction to form carnitine and acetyl-CoA to enter the tricarboxylic acid cycle (TCA) for energy production. This process is referred to as the carnitine shuttle. In addition, this shuttle also plays a role when yeast cells are grown on other non-fermentable carbon sources, such as acetate and ethanol. The metabolism of these compounds results in the production of acetyl-CoA in the cytoplasm, which needs to be transported to the mitochondria for energy production (Schmalix and Bandlow 1993; Stemple *et al*. 1998).

The existence of two pathways for the utilisation of peroxisomal acetyl-CoA was suggested on the basis of results showing that disruption of either the *CIT2* gene, encoding the peroxisomal glyoxylate cycle enzyme citrate synthase, or the *CAT2* gene,

encoding the peroxisomal and mitochondrial carnitine acetyltransferase, did not affect the growth of yeast on oleate, whereas a mutant with both genes disrupted (Δ*cit2*Δ*cat2*) failed to grow on this carbon source, due to an inability to oxidise this fatty acid (Van Roermund *et al.* 1995).
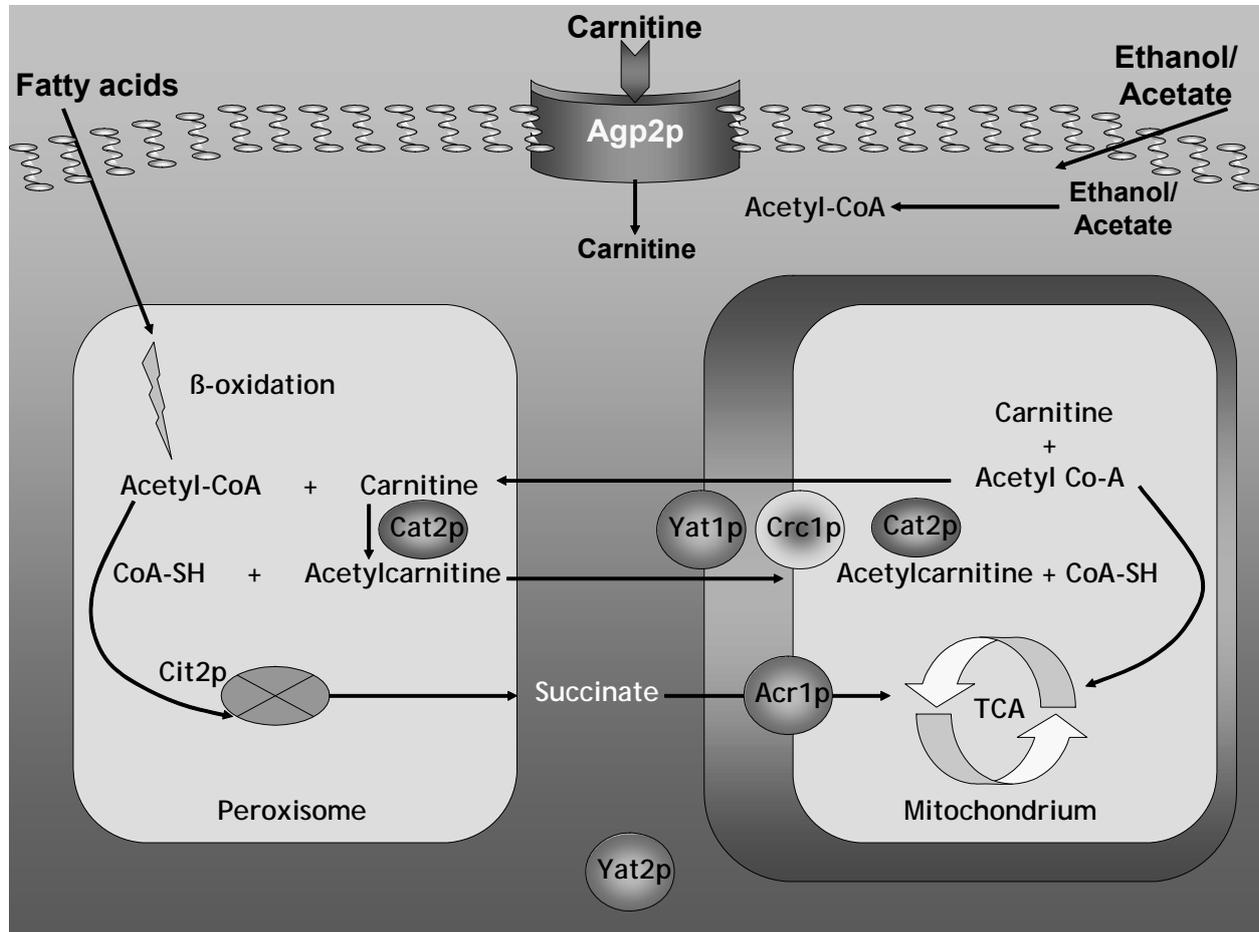
Figure 3.1. Schematic representation of the glyoxylate pathway and the carnitine shuttle in *Saccharomyces cerevisiae.* The three yeast carnitine acetyl transferases, Cat2p in the mitochondria and peroxisome, Yat1p on the outer-mitochondrial membrane and Yat2p in the cytosol are indicated along with the carnitine/acetylcarnitine translocase Crc1p. Cit2p combines two units of peroxisomaly generated acetyl-CoA, the fist step of the glyoxylate cycle, forming succinate.

Besides Cat2p, two additional CATs have been identified in *S. cerevisiae.* Cat2p is responsible for >95% of the total CAT activity in oleate-grown yeast cells (Kispal *et al.* 1993). A second gene, *YAT1*, codes for a CAT that presumably is associated with the outer surface of the mitochondria and contributes an estimated 5% of total CAT activity in acetate- and ethanol-grown cells (Schmalix and Bandlow 1993). A third gene, *YAT2,* codes for a CAT that has been suggested to be cytosolic and that shows a high contribution to CAT activity in ethanol-grown cells (Swiegers *et al.* 2001). The sequence homologies among the three CAT-encoding genes are extensive. Swiegers *et al.* (2001)

showed that, in a strain with a disrupted peroxisomal citrate synthase, all three carnitine acetyltransferases are essential for growth on all non-fermentable carbon sources.

This rather surprising set of data raises the question about the specific function of each of the three CAT enzymes in *S. cerevisiae*, since our current understanding of the carnitine shuttle does not require the existence of three independent carnitine acetyltransferases to ensure survival on non-fermentable carbon sources.

The role of carnitine in the metabolism of S. cerevisiae has not been investigated beyond the shuttling of acetyl residues. However, data in mammalian systems suggests that carnitine does have broader functions, such as its role in the maintenance of nutritional redox balance, the regulation of various gene subsets possibly involved in longevity and also regulation of the cells stress response (for review see Calabrese *et al*. 2006).

In this paper, we further explore specific roles of CAT enzymes and of carnitine. The data show that the three CAT enzymes play highly specific roles, since no cross complementation occurs even when individual CAT genes are overexpressed. Nevertheless, double and triple deletion mutants show phenotypes identical to the three single CAT gene deletion strains. The intracellular localisation of Cat2p and Yat1p confirms previous data, while we localise Yat2p to the cytoplasm by C-terminal GFP tagging. Our results also indicate that both carnitine and acetylcarnitine promote growth in a Δ*cit2* strain, and that this effect is dependent on the presence of all three CATs. Furthermore, the constitutive expression of the *Schizosaccharomyces pombe* malate permease (*MAE1*) gene can compensate for the growth defect of a Δ*cit2* strain when cells are grown on non-fermentable media supplemented with L-malic acid.

Since a role for carnitine as a stress protectant has been described in mammalian and bacterial systems (Kunau *et al*. 1995; Calabrese *et al*. 2006), the possibility of a similar function in yeast was investigated. Our data clearly show that the presence of carnitine improves growth in the presence of H_2O_2 and of weak organic acids such as lactate. Surprisingly, this effect appears to be independent of the activity of the carnitine shuttle. The data also indicate that deletion of *CAT2* leads to a significant reduction in the survival of cells grown in respiratory conditions after exposure to oxidative stresses. This is the first report of carnitine protecting against certain stresses in *S. cerevisiae*. Similar impacts in mammalian systems have been reported, but no information regarding the underlying mechanisms has been published. *S. cerevisiae* may be a useful model for elucidating the molecular nature of this protective activity.

3.2. MATERIALS AND METHODS

3.2.1. YEAST STRAINS AND MEDIA

All yeast strains used in this study are derived from strain S288c genetic background and are listed in Table 3.1. Yeast were grown either on rich YPD (1% yeast extract, 2% peptone, 2% glucose) or on minimal YND media, containing 0.67% (w/v) yeast nitrogen base (YNB) without amino acids (DIFCO) and 2% (w/v) glucose supplemented with amino acids according to the specific requirements of the respective strains. Amino acids were added to concentrations specified by Ausubel *et al*. (1994). To study the phenotypical effect of overexpression of each carnitine acetyltransferase, two different

Table 3.1. Yeast strains used in this study.

Yeast strains	Relevant genotype	Sources and references
FY23	MATa leu2 trp1 ura3	Winston *et al.* 1995
FY23Δ*yat1*	MATa trp1 ura3 Δyat1::LEU2	Swiegers *et al.* 2001
FY23Δ*yat2*	*MATa trp1 ura3 Δyat2::LEU2*	Swiegers *et al.* 2001
FY23Δ*cat2*	*MATa trp1 ura3 Δcat2::LEU2*	Swiegers *et al.* 2001
FY23Δ*yat2*Δ*yat1*	*MATa ura3 Δyat2::LEU2 Δyat1::TRP1*	This study
FY23Δ*yat1*Δ*cat2*	*MATa trp1 Δyat1::LEU2 Δcat2::URA3*	This study
FY23Δ*yat2*Δ*cat2*	*MATa trp1 Δyat2::LEU2 Δcat2::URA3*	This study
FY23Δ*yat2*Δ*cat2*Δ*yat1*	*MATa yat2::LEU2 cat2::URA3 yat1::TRP1*	This study
FY23Δ*cit2*Δ*yat1*	*MATa ura3 Δcit2::TRP1 Δyat1::LEU2*	Swiegers *et al.* 2001
FY23Δ*cit2*Δ*yat2*	*MATa ura3 Δcit2::TRP1 Δyat2::LEU2*	Swiegers *et al.* 2001
FY23Δ*cit2*Δ*cat2*	*MATa ura3 Δcit2::TRP1 Δcat2::LEU2*	Swiegers *et al.* 2001
BY4747	*MATα his3 leu2 lys2 ura3*	Euroscarf deletion library
BY4747Δ*yap1*	*MATα his3 leu2 lys2 ura3 yap1::KanMX4*	Euroscarf deletion library
BY4747Δ*yat1*	*MATα his3 leu2 lys2 ura3 yat1::KanMX4*	Euroscarf deletion library
BY4747Δ*yat2*	*MATα his3 leu2 lys2 ura3 yat2::KanMX4*	Euroscarf deletion library
BY4747Δ*cat2*	*MATα his3 leu2 lys2 ura3 cat2::KanMX4*	Euroscarf deletion library

media with non-fermentable carbon sources were used. Both media contained 0.67% (w/v) YNB without amino acids (DIFCO), 2% of agar and either 2% (v/v) ethanol (YNE) or 2% (w/v) glycerol (YNG) as sole carbon source, supplemented with 10 mg/L of L-carnitine (+C).

For the localisation studies, yeast was grown in test tubes containing 5 ml of either YNE [0.67% (w/v) YNB without amino acids (DIFCO) and 2 % (v/v) ethanol] or YNO [0.67% (w/v) YNB without amino acids (DIFCO) and 0.1 % oleic acid] media. To investigate the effect of the *Schizosaccharomyces pombe* malate permease (*MAE1*) gene, strains were spotted on YNEM media, containing 0.67% (w/v) YNB without amino acids (DIFCO), 2% (v/v) ethanol, 1% (w/v) L-malic acid and 0.5% agarose, the pH of the media was set to 3.5 with 1M KOH. The effect of organic acid stress was monitored on YND media with various concentrations of acetate and lactate added, the pH was buffered at 3.5 with phosphate buffer to ensure that any differences observed would be due to the presence of the mentioned organic acids and not variation in pH. For the stress tolerance experiments, cells were either grown in YND or YNG media with addition of the required stress agent and carnitine where indicated. YNG was preferably used for all stress experiments, since growth on non-fermentable carbon sources utilises the carnitine shuttle, with the only exception being oxidative stress under growing conditions which was performed on YND plates containing H_2O_2, since YNG does not support growth in these conditions.

3.2.2. DNA MANIPULATION

All plasmids used in this study are listed in Table 3.2 and the primers are listed in Table 3.3. Standard DNA techniques were carried out as described by Sambrook *et al*. (1989). Standard procedures for the isolation and manipulation of DNA were used throughout the study (Ausubel *et al*. 1994). Restriction enzymes, T4 DNA-ligase and Expand Hi-Fidelity polymerase used in the enzymatic manipulation of DNA were obtained from Roche Diagnostics (Randburg, South Africa) and used according to the specifications of the supplier. *Escherichia coli* DH5α (GIBCO-BRL/Life Technologies) was used as host for the construction and propagation of all plasmids. Sequencing of all plasmids was carried out on an ABI PRISM^{TM} automated sequencer.

Table 3.2. Clones and constructs.

Plasmids	Relevant genotype	Sources and references
YCplac33	*CEN4 URA3*	Gietz and Sugino 1988
YEplac112	*2μ TRP1*	Gietz and Sugino 1988
YEplac181	*2μ LEU2*	Gietz and Sugino 1988
Ydp-W	*TRP1*	Berben *et al*. 1991
Ydp-U	*URA3*	Berben *et al*. 1991
pGEM-T-easy-*YAT1*		Swiegers *et al*. 2001
pGEM-T-easy-*YAT2*		Swiegers *et al*. 2001
pGEM-T-easy-*CAT2*		Swiegers *et al*. 2001
pΔ*cat2*	Δ*cat2::URA3*	This study
pΔ*yat1*	Δ*yat1::LEU2*	Swiegers *et al*. 2001
pΔ*yat1*	Δ*yat1::TRP1*	This study
YEplac112-T-GFP2-B-BGL	*2μ TRP1*	Our laboratory
pYES2-mtBFP	*2μ URA3*	Westermann and Neubert 2000
YCplac33-*YAT1*-GFP	*CEN4 URA3 YAT1*	This study
YCplac33-*YAT2*-GFP	*CEN4 URA3 YAT2*	This study
YCplac33-*CAT2*-GFP	*CEN4 URA3 CAT2*	This study
pGEM-T-easy-Cat2p		This study
YEplac 112-mtBFP	*2μ TRP1*	This study
YEplac181-BFP-P	*2μ LEU2*	This study
YCplac33-PGKpt	CEN4 *URA3 PGK*$_P$ *PGK*$_T$	Our laboratory
YCplac33-PGKp-*YAT1*-PGKt	*CEN4 URA3 YAT1*	This study
YCplac33-PGKp-*YAT2*-PGKt	*CEN4 URA3 YAT2*	This study
YCplac33-PGKp-*CAT2*-PGKt	*CEN4 URA3 CAT2*	This study
pHV3	*2μ LEU2 PGK*$_P$ *MAE1 PGK*$_T$	Volschenk *et al*. 1997
YCplac33-PGKp-*MAE1*-PGKt	*CEN4 URA3 MAE1*	This study

Table 3.3. Primers used in this study. Introduced primer sequences are underlined.

Primer	Sequence
CAT2F1	5'-GACACTGTTCGCCAAATTTCG-3'
CAT2R1	5'-ATAAGCAAGGCACAATATCC-3'
YAT1F1	5'-ATCAGCATCAGCATCAGC-3'
YAT1R1	5'-AGAGGTAATCCAAACGACG-3'
YAT1-GFP-F	*5'-GATC<u>GAATTC</u>GTGGAAATCATCGCGCGCAAGCCA-3'
YAT1-GFP-R	*5'-GATC<u>GGTACC</u>ACCGGACACGCTCACGTCGAAGTA-3'
YAT2-GFP-R	*5'-GATC<u>GGTACC</u>TTGATCTAAGGTCGCCACCTTTCT-3'
YAT2-GFP-F	*5'-GATC<u>GAATTC</u>GAGGCAGCCCGTGTTGCGTCACAA-3'
CAT2-GFP-F	*5'-GATC<u>GAATTC</u>TTTCTTGGAAATTCTGTCAAATCT-3'
CAT2-GFP-R	*5'-GATC<u>GGTACC</u>TAACTTTGCTTTTCGTTTATTCTC-3'
Cat2p	5'-GATC<u>CTGCAGTCGCGA</u>GAGTGCTTTCTTTTTAG-3'
BFP-P(R)	*5'-GATC<u>AAGCTT</u>TTA*TAACTTTGC*TTTGTATAGTTCATCCATGCCAT-3'
BFP-P(F)	*5'-GATC<u>TCGCGA</u>ATGAGTAAAGGAGAAGAACTTTTCAC-3'
mtBFP-F	*5'-GATC<u>TCGCGA</u>ATGGCCTCCACTCGT-3'
mtBFP-R	*5'-GATC<u>AAGCTT</u>TTATTTGTATAGTTCATCCATGCCATGT-3'
CAT2ov-F	*5'-GATC<u>GAATTC</u>ATGAGGATCTGTCATTCGA-3'
CAT2ov-R	*5'-GATC<u>CTCGAG</u>TCATAACTTTGCTTTTCG-3'
YAT1ov-F	*5'-GATC<u>GAATTC</u>ATGCCAAACTTAAAGAGACT-3'
YAT1ov-R	*5'-GATC<u>CTCGAG</u>TCAACCGGACACGCTCA-3'
YAT2ov-F	*5'-GATC<u>GAATTC</u>ATGTCAAGCGGCAGTA-3'
YAT2ov-R	*5'-GATC<u>GTCGAC</u>TTATTGATCTAAGGTCGCC-3'
5'-mae1	*5'-GATC<u>GAATTC</u>ATGGGTGAACTCAAGGAAATC-3'
3'-mae1	*5'-GATC<u>AGATCT</u>TTAAACGCTTTCATGTTCACT-3'

3.2.3. CONSTRUCTION OF MULTIPLE CAT MUTANTS

To create the double mutants FY23Δ*yat1*Δ*cat2* and FY23Δ*yat2*Δ*cat2*, the *CAT2* gene was disrupted in either the FY23Δ*yat1* or FY23Δ*yat2* strain (Swiegers *et al*. 2001). For

this purpose, 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, and this was transformed into FY23Δ*yat1* and FY23Δ*yat2.* Transformants were isolated on selective media and the disruption was verified by polymerase chain reaction (PCR) using the primers CAT2F1 and CATR1 (Table 3). To create the FY23Δ*yat1*Δ*yat2* double mutant, a Δ*yat1* disruption construct was created by using the plasmid pΔ*yat1*::*LEU2* and removing the *LEU2* gene 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. This was transformed into strain FY23Δ*yat2* and the disruption was verified using primers YAT1F1 and YAT1R1. To create a yeast strain without any known CAT-encoding genes, the same fragment was transformed into strain FY23Δ*yat2*Δ*cat2*. The disruptions were verified using primers YAT1F1 and YAT1R1.

3.2.4. CONSTRUCTION OF CAT-GFP AND BFP PLASMIDS

The 759 bp GFP open reading frame contains a *Kpn*I site nine nucleotides after the start codon. The *YAT2* gene was amplified from the pGEM-T-easy-*YAT2* plasmid using the primers YAT2-GFP-F and YAT2-GFP-R. In the reverse primer, the stop codon was replaced with a *Kpn*I restriction site (Table 3). The resulting 3.1 kb fragment was inserted as a *Eco*RI/*Kpn*I fragment into the YEplac112-T-GFP2-B-BGL plasmid. From this construct, a 4.0 kb *Xba*I/*Nar*I fragment was subcloned into the YCplac33 vector. The *YAT1* and *CAT2* genes and their respective promoter regions were amplified from the pGEM-T-easy-*YAT1* or pGEM-T-easy-*CAT2* plasmid, using either the YAT1-GFP-F and YAT1-GFP-R or the CAT2-GFP-F and CAT2-GFP-R primers. The resulting *Eco*RI/*Kpn*I fragments of 3.1 kb were subsequently ligated in the YCplac33-*YAT2-GFP* plasmid digested with *Eco*RI and *Kpn*I containing the GFP open reading frame.

For co-localisation studies, the blue fluorescent protein (BFP) encoding gene, with either a mitochondrial or a peroxisomal signal sequence, was used and expressed under the promoter of *CAT2*. The promoter was amplified from the YCplac33-*CAT2-GFP* plasmid by using the primers CAT2-GFP-F and Cat2p. The resulting 1.2 kb fragment was blunt-ligated into the pGEM-T-easy vector and digested with *Eco*RI and

*Pst*I, purified and subcloned into YEplac181 and YEplac121. To fuse the mitochondrial-targeted blue fluorescent protein to the promoter, the BFP and the mitochondrial pre-sequence were amplified by PCR, using the mtBFP-F and mtBFP-R primers and the pYES2-mtBFP plasmid as template. The resulting 960 base pair fragment was cloned into the plasmids as a *Nru*I/*Hind*III digest. To construct the peroxisomal-targeted BFP, a primer was designed containing the peroxisomal targeting signal type 1 (PTS-1) at the C-terminus of the open reading frame (Table 3). Using the primers BFP-P(R) and BFP-P(F), a 630 base pair fragment was obtained by PCR, using the pYES2-mtBFP plasmid as template, and cloned as a *Nru*I/*Hind*III fragment into the YEplac181 and YEplac121 vectors containing the *CAT2* promoter.

3.2.5. CONSTRUCTION OF THE OVEREXPRESSION PLASMIDS

The *CAT2* gene was amplified from the YCplac33-*CAT2-GFP* plasmid template by PCR, using primers CAT2ov-F and CAT2ov-R (Table 3). The resulting 2.0 kb fragment was blunt-ligated into the pGEM-T-easy vector. The *YAT1* gene was amplified from the YCplac33-*YAT1-GFP* plasmid template by PCR, using the YAT1ov-F and YAT1ov-R primers. A 2.1 kb fragment was ligated into the pGEM-T-easy vector. The remaining carnitine acetyltransferase gene, *YAT2*, was amplified from the YCplac33-*YAT2-GFP* plasmid template by PCR using the YAT2ov-F and YAT2ov-R primers. A 2.7 kb fragment was ligated into the pGEM-T-easy vector. The pGEM-T-easy-*CAT2* and pGEM-T-easy-*YAT1* plasmids were digested with *Eco*RI and *Xho*I, resulting in fragments of 2037 and 2104 base pairs respectively. The pGEM-T-easy-*YAT2* plasmid was digested with *Eco*RI and *Sal*I and resulted in a fragment of 2796 base pairs. These fragments were ligated into the *Eco*RI/*Xho*I site of the YCplac33-*PGK*pt plasmid. The resulting three plasmids, YCplac33-*PGK*pt-*CAT2,* YCplac33-*PGK*pt-*YAT1* and YCplac33-*PGK*pt-*YAT2*, contained the *CAT2, YAT1* or *YAT2* gene under control of the phosphoglycerate kinase I (*PGK*) promoter.

3.2.6. CONSTRUCTION OF THE MAE1 EXPRESSION PLASMID

The *Schizosaccharomyces pombe* malate permease gene, *MAE1*, was amplified from genomic DNA by PCR, using primers 5'-mae1 and 3'-mae1 (Table 3) and blunt-ligated into the pGEM-T-easy vector. This plasmid was subsequently digested with *Eco*RI and

*Bgl*II and the resulting fragment of 1323 base pairs, corresponding to the open reading frame of the *MAE1* gene, was then ligated into the *Eco*RI/*Bgl*II site of the YCplac33-PGKpt plasmid.

3.2.7. FLUORESCENT MICROSCOPY

In order to determine the intracellular location of the three proteins Yat1p, Yat2p and Cat2p in vivo, each was C-terminally tagged with GFP. Centromeric shuttle vectors carrying in-frame fusions of GFP to the 3' end of *YAT1*, *YAT2* and *CAT2* under transcriptional control of their respective native promoters were introduced into the haploid yeast FY23. For direct fluorescent visualisation of the yeast peroxisomes and mitochondria, both organelles were targeted with a BFPp controlled by the promoter of *CAT2* on a multi-copy plasmid. A Nikon E400 microscope with UV source and appropriate filter sets was used to visualise fluorescence. Images were taken with a Nikon COOLPIX 990 digital camera. Scion Image for Windows was used to capture video images, and Microsoft Photo Editor 3.0 was used for editing the images.

3.2.8. STRESS TOLERANCE EXPERIMENTS

The osmotic stress experiments were performed by spotting serial dilutions (1:10) of cultures of the wild type strains BY4742 and FY23 and also the various single double and triple mutants of the CATs on YND media with various concentrations of sodium chloride up to 2 M and also on the same series of media with carnitine added to 10 mgL^{-1}. In order to confirm the results quantitatively, growth curves were performed in liquid media with the same strains under similar NaCl concentrations.

For the evaluation of oxidative stress tolerance, pre-cultures of cells were grown overnight to be inoculated into fresh YND media. The cultures were grown to an optical density (O.D.), measured at 600 nm, of ~ 1 and diluted to a concentration of approximately 3×10^6 cells per ml. Five subsequent 10:1 dilutions were spotted in 10 µL droplets onto YND media containing 0.5, 1, 1.5 and 2 mM of hydrogen peroxide with and without addition of L-carnitine (1, 10, 100 and 1000 mgL-1). The plates were incubated at 30°C for three days after which growth was monitored.

To perform oxidative and thermal stress experiments under non-growing conditions cells from overnight cultures were inoculated into YNG media with and without carnitine

added (1, 10, 100 and 1000 mgL^{-1}) and grown to mid-log phase (O.D.600 of ~ 1). Cultures were washed once in 0.9% NaCl, diluted to a concentration of ~ 2 X 10^6 cells per ml in 0.9% NaCl containing 0.1% of Triton-X100 and 3 mM of hydrogen peroxide for the induction of oxidative stress. At measured time intervals 100 μL of cells were removed and appropriate dilutions were plated onto YPD an incubated at 30°C for two days after which the plated colonies were counted.

Cultures used for thermal stress experiments were treated similarly. Cells were harvested, washed once and resuspended in sterile water at a concentration of approximately 3 X 10^7 cells per ml. Aliquots of 100 μl were dispensed into 0.6 ml PCR tubes and incubated at 45°C for 45 min in a thermocylcer. For the induction of thermotolerance, cells were pre-incubated at 40°C for 30 min and subsequently treated at 50°C for 45 min. At different time intervals cells were removed, placed on ice for one minute to be plated onto YPD in appropriate dilutions. The plates were incubated for two days. The percentage of survival was determined against untreated cells. Differences between assay values were calculated at a $p < 0.05$ level using the student paired t-test.

Similarly, strains to be used in the organic acid tolerance experiments were grown in YND to an O.D.$_{600}$ of ~ 1, washed once with sterile water and diluted to a concentration of approximately 3 X 10^6 cells per ml. Aliquots of 10 μl of the resuspended cells and also four subsequent 1:10 dilutions were spotted on YND plates containing 2.1, 4.2, or 5.1 % v/v of lactate or 0.2, 0.3, 0.4 % v/v of acetate, with and without the addition of carnitine to a final concentration of 10 mgL^{-1}. Plates were monitored for growth after 72 hours incubation at 30°C.

3.3. RESULTS

3.3.1. EFFECT OF CAT GENE DELETION ON CELLULAR GROWTH

To monitor if single CAT gene deletions or combination of deletions in double and in the triple disrupted strains have an effect on yeast growth, growth curves were obtained from cultures grown in fermentable (YND) and non-fermentable (YNE) media supplemented with L-carnitine. As shown in Figure 3.2, no significant difference in growth characteristics could be observed. Duration of lag phase, mean generation time in the exponential growth phase and the final biomass production were similar. It also is

clear, that in strains with a functional peroxisomal citrate synthase, the inability to shuttle acetyl-CoA via the carnitine system does not affect the growth speed, since the mean generation time of the strains in YNE media with and without supplementation of L-carnitine showed no significant differences. Growth in other non-fermentable carbon sources and also oleate followed the same pattern (data not shown).
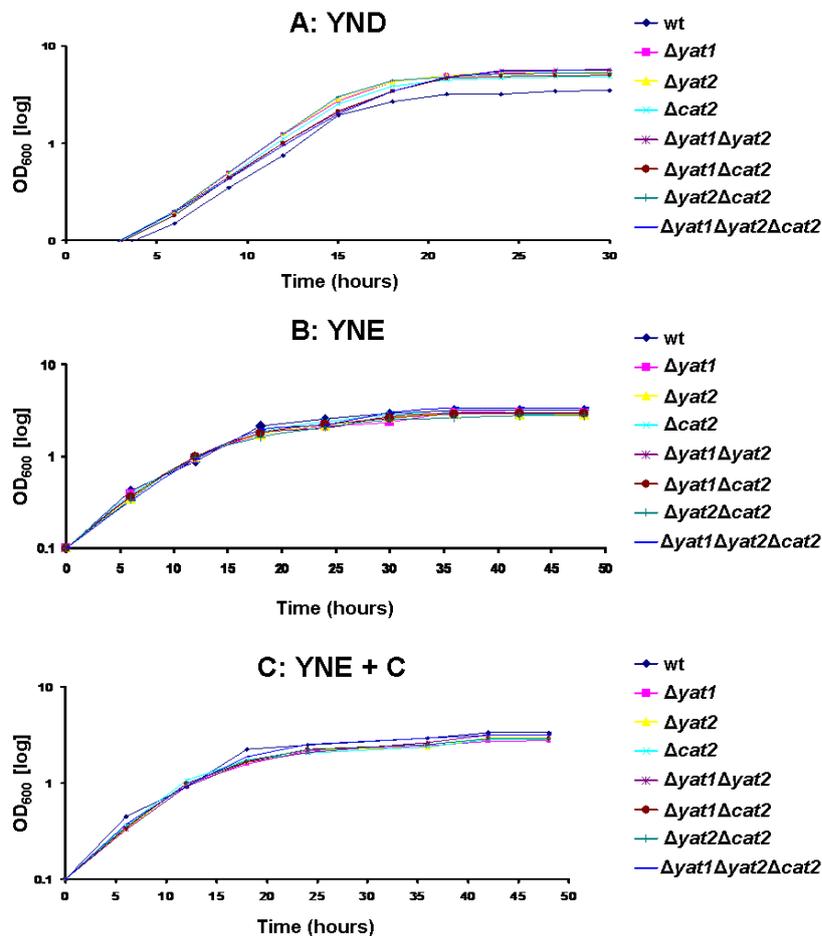
Figure 3.2. Growth curves of single, double and triple CAT gene deletions grown in (**A**) YND media, (**B**) YNE media and (**C**) YNE media supplemented with L-carnitine.

3.3.2. SUB-CELLULAR LOCALISATION OF THE THREE CARNITINE ACETYL-TRANSFERASES

In order to determine the exact intracellular localisation of each CAT protein (Yat1p, Yat2p and Cat2p) in vivo, each was C-terminally tagged with GFP. Wild type strain FY23 was transformed with the centromeric plasmid YCplac33, carrying in-frame fusions of GFP to the 3' end of *YAT1*, *YAT2* and *CAT2* under transcriptional control of their respective native promoters. To differentiate between cellular organelles, e.g. mitochondria and peroxisomes, strains were co-transformed with one of the shuttle

vectors YEplac112 and YEplac181, carrying the BFP with either a mitochondrial or a peroxisomal signal sequence under the transcriptional control of the *CAT2* promoter.

Figure 3.3 shows the blue fluorescent protein that was used as a control to distinguish between the mitochondria and the peroxisome and also the three GFP tagged CATs. In all co-transformed strains, similar fluorescent signals could be observed for the targeted BFP.
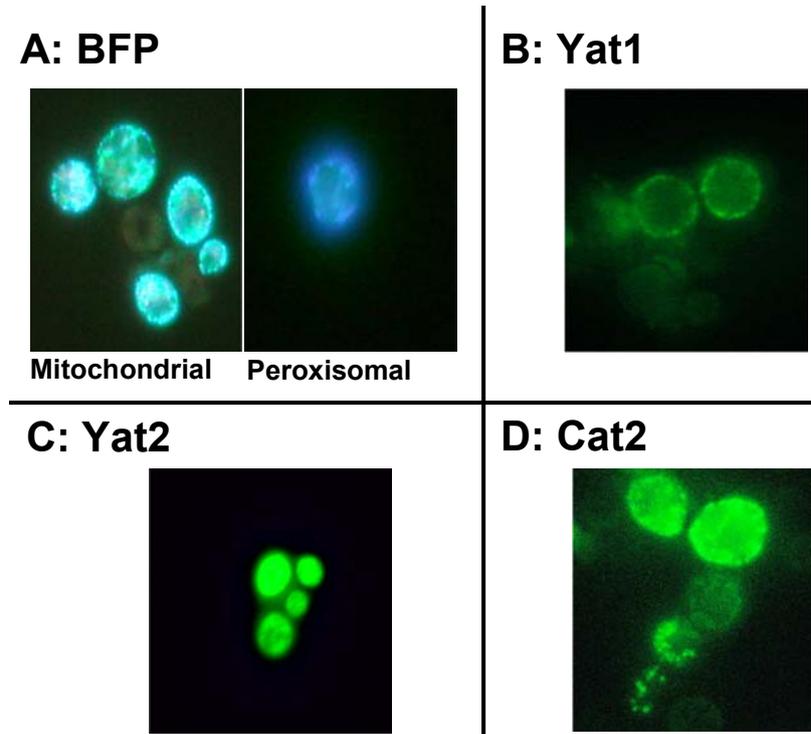
Figure 3.3. Localization of the three yeast CATS. (**A**) BFP tagged with either a peroxisomal or mitochondrial signaling sequence which was used as a control and co-expressed with GFP tagged versions of (**B**) *CAT2*, (**C**) *YAT2* or (**D**) *YAT1*. Signals generated by the GFP chimeras concur with co-expressed BFP localized to either the mitochondria for Yat1p and the mitochondria and peroxisome in the case of Cat2p. Yat2p-GFP is visualised throughout the cytosol.

Yat1p is linked to the mitochondria, which confirms existing data. However, the fluorescent signal is much weaker than for Cat2p. Cat2p is associated with the mitochondria and what is presumably the peroxisome. Yat2p, on the other hand, appears to be distributed throughout the cell, suggesting cytoplasmic localisation.

3.3.3. COMPLEMENTATION OF THE GROWTH DEFECT OF THE Δ*cit2* MUTANT THROUGH THE CARNITINE SHUTTLE

Previous studies have indicated that deletion of *CIT2* creates a strain that is completely dependent on the presence of carnitine for growth on non-fermentable carbon sources

and fatty acids. In these conditions the activities of all three CATs are required and deletion of any one leads to a loss of growth even in the presence of carnitine (Swiegers *et al*. 2001). Supplementation of media with either carnitine or acetylcarnitine was able to rescue the Δ*cit2* growth defect (Figure 3.4), while deletion of any one of the CATs in addition to *CIT2* results in loss of growth.
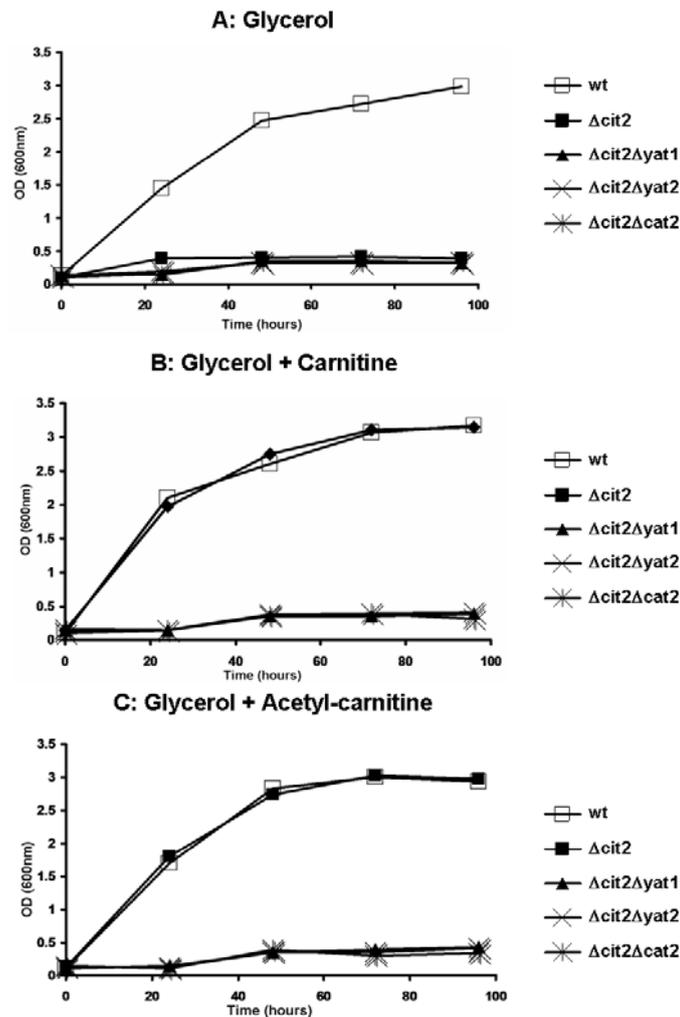
Figure 3.4. Growth of Δ*cit2* and Δ*cit2*Δcat mutant strains in (**A**) YNG, (**B**) YNG + carnitine and (**C**) YNG +acetylcarnitine, both supplemented to 100 mgL^{-1}.

To assess if the overexpression of one of the three CAT genes under the constitutive PGK promoter could overcome the growth defect on non-fermentable carbon sources in the Δ*cit2* background, the double mutants (FY23Δ*cit2*Δ*yat1*; FY23Δ*cit2*Δ*yat2* and FY23Δ*cit2*Δ*cat2*) were transformed with the YCplac33-PKGpt plasmid carrying, either the *YAT1*, *YAT2* or *CAT2* gene. Transformants were streaked out on YNE or YNG agar plates, supplemented with L-carnitine. The plates were incubated at 30°C for 14 days. In the Δ*cit2* background, the overexpression of the deleted CAT gene could rescue

growth (Figure 3.5 A-C), indicating the functionality of the genes on the plasmid. However, in none of the cases cross-complementation could be observed, indicating a specific and unique metabolic function for each of the three CAT genes.
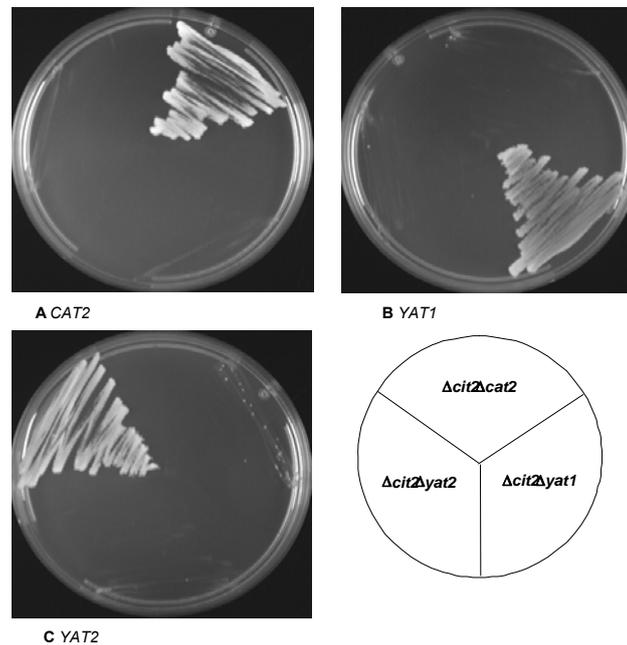
Figure 3.5. Overexpression of the CAT genes under the *PGK1* promoter. Strain layout as indicated. (**A**) overexpression of *CAT2*, (**B**) overexpression of *YAT1* and (**C**) overexpression of *YAT2*. Overexpression of each of the CAT genes was able compliment its own deletion, but unable to cross-complement deletion of any of the other two CATs.

3.3.4. EXPRESSION OF S. *pombe MAE1* COMPENSATES FOR THE Δ*cit2* GROWTH DEFECT ON NON-FERMENTABLE CARBON SOURCES

Peroxisomally produced acetyl-CoA can be utilised in two ways, either through shuttling via the carnitine system, or by forming C_4 compounds, in particular succinate by the glyoxylate cycle. To assess whether efficient uptake of extracellular C_4 compounds, which is not present in *S. cerevisiae*, could compensate for the glyoxylate cycle and possibly induce phenotypical differences between the three CAT mutants, we generated a strain that has an efficient uptake of C_4 dicarboxylic acid by expressing the *S. pombe* malate permease gene (*MAE1*). For this purpose, the various Δ*cit2* mutant strains were transformed with YCplac33-PKGpt plasmid carrying the *MAE1* gene or the YCplac33-PKGpt plasmid as control. Strains were grown in YND media to an optical density (OD_{600}) of ~ 1.0 and then spotted in five 10:1 serial dilutions on YNEM media with and without carnitine. The plates were incubated at 30°C for 7 days. As shown in Figure 3.6, the expression of the *S. pombe* malate permease (*MAE1*) gene complemented the

carnitine dependency of a Δ*cit2* deletion strain. Furthermore, expression of *MAE1* complements deletion of *CIT2* in combination with any of the three CATs, while no difference is observed between the three Δ*cit2*Δ*cat* double mutants.

Addition of carnitine however did not only results in the complementation of Δ*cit2*, but also led to significantly enhanced growth of the wild type strain FY23 on YNEM. This is the first carnitine-dependent phenotype observed in a WT genetic background. The enhancement of growth by carnitine is especially clear in strains expressing the *MAE1* gene, even when CAT genes are deleted.
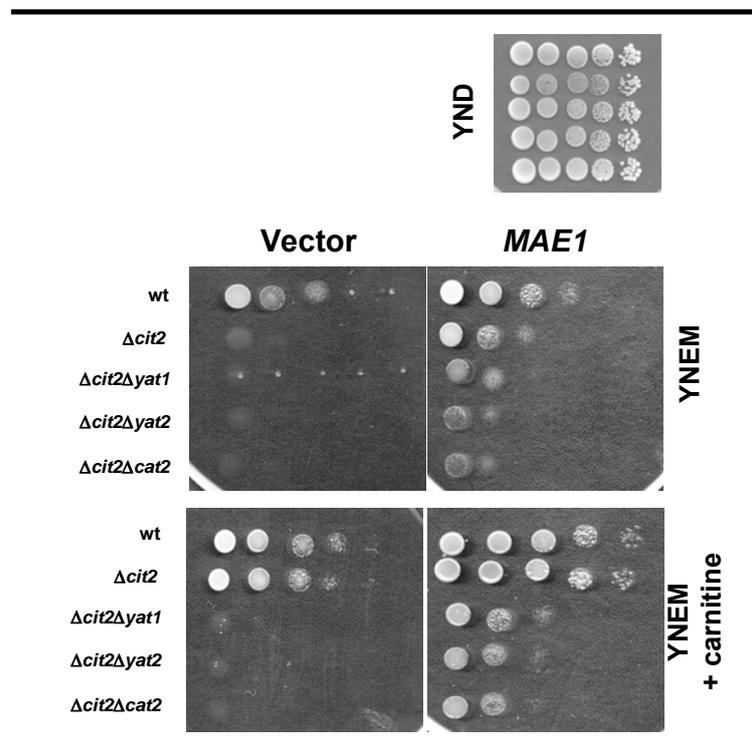
Figure 3.6. Serial dilutions of the wild type strain, the Δ*cit2* background and the Δ*cit2*Δ*cat* double mutants expressing the *Schizosaccharomyces pombe* malate permease gene (*MAE1*). The growth medium used was YNEM and also YNEM containing carnitine. Similar to the addition of carnitine, overexpression of *MAE1* rescues the growth defect of a Δ*cit2* strain in these conditions. Carnitine addition further enhances growth of the wild type, FY23, and also the mutant strains on YNEM plates, especially in strains expressing *MAE1*.

3.3.5. CARNITINE ENHANCES GROWTH DURING ORGANIC ACID STRESS INDUCED BY THE PRESENCE OF LACTATE

These results led us to investigate a possible role of carnitine in alleviating organic acid toxicity in yeast. For this purpose, growth was monitored on plates containing harmful concentrations of lactic and acetic acid, as previously described (Kawahata *et al*. 2006).

Carnitine addition to YND plates containing lactate results in an increase in growth compared to the plates without carnitine (Figure 3.7). This effect was very clear on plates containing 5.1% lactate. This enhancement is similar to what is observed on the malate containing YNEM plates (Figure 3.6). Furthermore, the effect of carnitine was independent of the presence of the three CATs and therefore also of the carnitine shuttle. A similar phenotype could, however, not be seen in the case of acetate containing plates (data not shown).
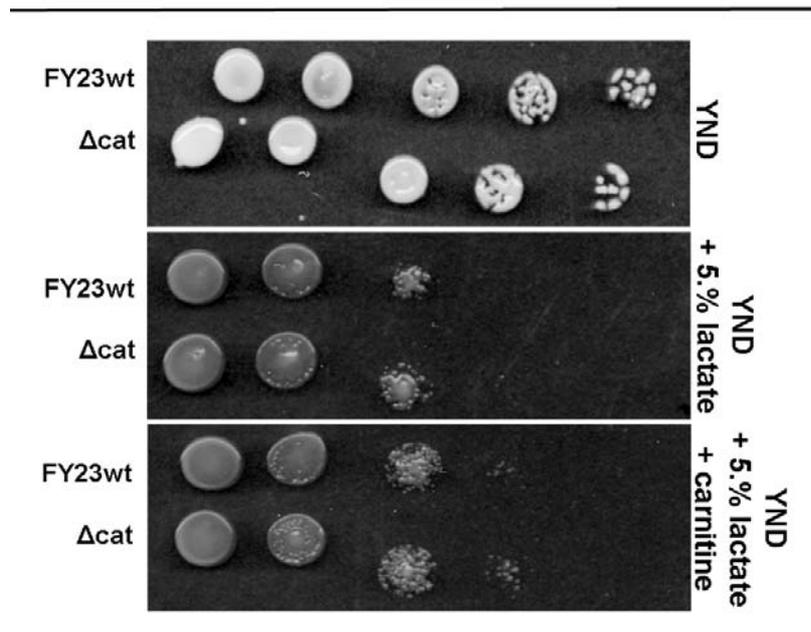
Figure 3.7. Serial dilutions of the wild type strain FY23 and the triple CAT mutant FY23Δ*yat1*Δ*yat2*Δ*cat2* (indicated as Δcat) spotted on YND plates containing 5.1% lactate with and without the addition of carnitine. Carnitine enhances growth under conditions of organic stress induced by the presence of lactate, independent of the carnitine shuttle.

3.3.6. CARNITINE PROMOTES GROWTH IN THE PRESENCE OF HYDROGEN PEROXIDE

To further investigate the possible role of carnitine in stress protection, we assessed various other stress conditions. Studies in bacterial systems have established that carnitine can act in a protective capacity as a compatible solute in osmotic stress conditions in prokaryotes (Kunau *et al*. 1995). In humans the emerging role of carnitine and acetylcarnitine as therapeutic agents for neurodegenerative disorders and anti-oxidant modulator has recently been speculated to involve the redox dependent activation of vitagenes through induction of the heat shock response. Induction of the heat shock response has also been suggested in protection against various diseases,

such as cancer aging and also various states of neurodegeneration (for review see Calabrese *et al*. 2006).

In order to investigate a possible role for carnitine in protection against conditions of cellular stress in yeast, cells were subjected to osmotic (NaCl), thermal and oxidative (H_2O_2) stresses. In the case of osmotic, thermal and oxidative shock in non-growing conditions no difference in survival was observed between cells grown in the presence or absence of carnitine (Figure 3.8, A-D), which was added at concentrations ranging from 1 to 1000 mgL^{-1} (only data for 100 mgL^{-1} carnitine are shown). Carnitine does, however, clearly enhance growth of the wild type strain BY4742 in the presence of hydrogen peroxide (Figure 3.8 E). Growth enhancement was independent of carnitine concentrations (1, 10, 100 and 1000 mgL^{-1}). This finding was also confirmed in the FY23 genetic background. Furthermore, addition of carnitine does not change the requirement for the transcriptional activator Yap1p (Figure 3.8, E) which was used as control.
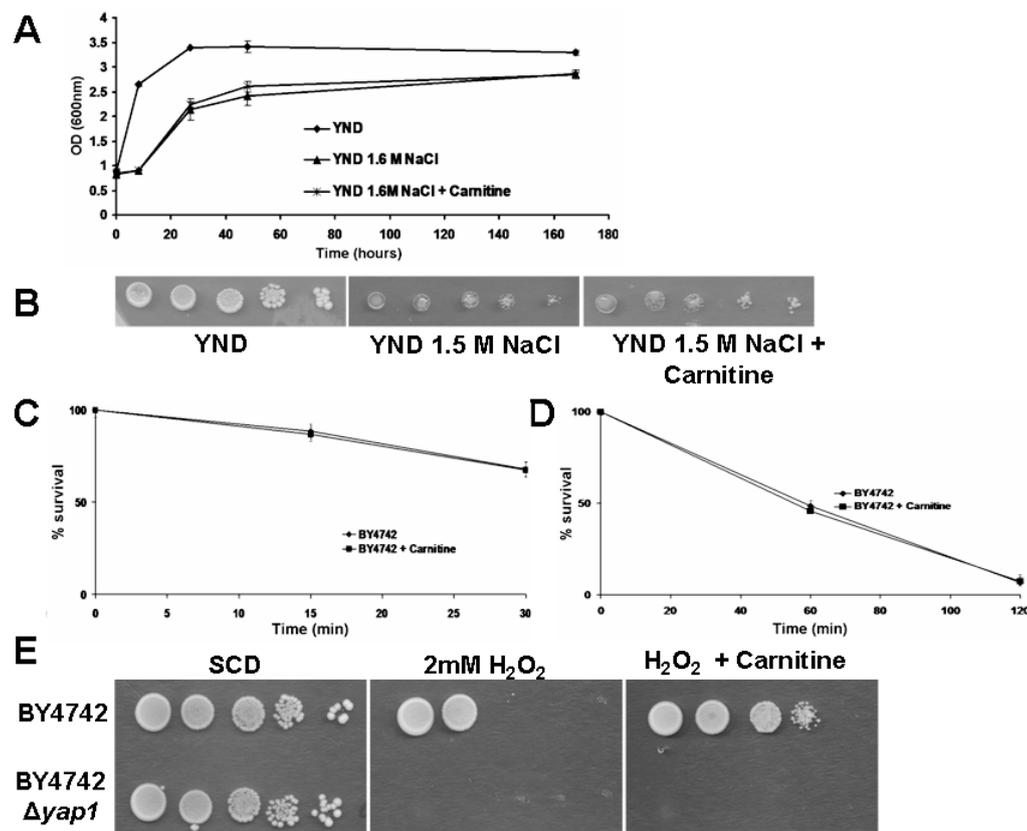
Figure 3.8. Stress tolerance experiments performed on the wild type BY4742 strain. (**A**) Growth curve of strains in YND compared to YND with 1.6 M NaCl and carnitine addition. (**B**) Serial 1:10 dilutions spotted onto plates containing 1.5 M NaCl. (**C**) Percentage survival after exposure to 45°C, strains was harvested from YNG cultures with and without carnitine addition. (**D**) Percentage survival after exposure to 3 mM H_2O_2, strains was harvested from YNG cultures with and without carnitine addition. (**E**) Serial dilutions of BY4742 and BY4742Δ*yap1* spotted onto plates containing 2 mM H_2O_2. All experiments were done in triplicate.

3.3.7. *CAT2* IS REQUIRED FOR PROTECTION AGAINST OXIDATIVE SHOCK IN CELLS GROWN UNDER RESPIRATORY CONDITIONS

To further investigate whether the carnitine shuttle or the three yeast CATs are involved in or have an impact on the yeast's ability to tolerate various cellular stresses, strains of the BY4742 genetic background with deletions of each CAT gene were exposed to thermal, oxidative and osmotic stress conditions. In the case of thermal stress, we also assessed resistance after a period of pre-adaptation by heating the cells to 40°C for 30 min before a thermal shock was executed at 50°C. In the case of oxidative stress on YND plates (performed as described for the wild type strains) no difference could be observed between the mutant strains (data not shown). There was also no difference in the survival of the strains exposed to thermal and osmotic shock (data not shown). However, the survival percentage after exposure to oxidative stress and also thermal stress with pre-adaptation (Figure 3.9, A and B), was reduced by 10% to 20% in the Δ*cat2* mutant strain when compared to the wild type. This reduced survival was independent of the presence of carnitine in the culture growth medium. No difference could be observed between the Δ*yat1* and Δ*yat2* mutant strains and the wild-type.
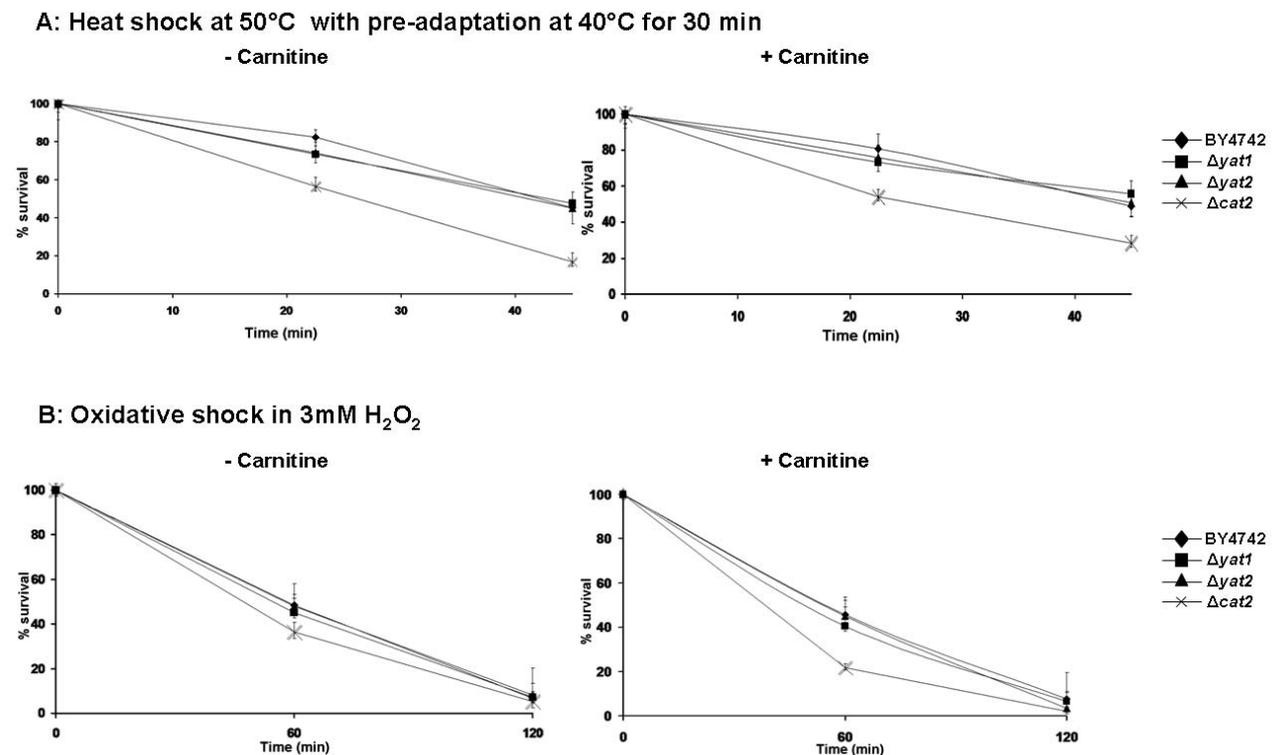
Figure 3.9. Survival percentages of BY4742 and strains bearing single mutations of each CAT after exposure to (**A**) heat shock at 50°C with a 30 min period of pre-adaptation at 40°C and (**B**) Oxidative shock in 3 mM H_2O_2. Strains were grown in YNG media with and without carnitine addition prior to induction of stresses. All experiments were done in triplicate.

3.4. DISCUSSION

The aim of this study was to investigate the specific roles of the three CAT genes encoded by the yeast genome and to identify possible physiological roles of L-carnitine. Our genetic analysis of CAT and *CIT2* deletion mutants in all combinations clearly demonstrate that the shuttle system has no effect on growth in strains with a functional glyoxylate cycle.

The data also confirm previous information regarding the localization of Cat2p and Yat1p, and localise Yat2p to the cytosol. The overexpression of any of the three CAT genes can not cross-complement the deletion of another CAT gene, indicating that each of the genes fulfils a unique and very specific function in the shuttling and regulation of acetyl-CoA and CoA pools inside the various cellular compartments. It is unlikely that the specificity is only due to the difference in localisation. Indeed, in such a case, some cross complementation in overexpressing strains would be likely. In addition, it is unclear why three enzymes would be required to maintain a "minimum "shuttling activity if such activity was limited to the transfer of activated acetyl residues between cytoplasm, mitochondria and peroxisomes.

Furthermore, addition of either carnitine or acetylcarnitine to the growth medium reinstates growth in the carnitine dependent Δ*cit2* mutant strain, indicating that yeast cells are able to take up acetylcarnitine from the growth medium.

The provision of C_4 compounds through the expression of the *Schizosaccharomyces pombe* malate permease gene (*MAE1*) was able to compensate for the growth defect of the Δ*cit2* deletion strains on non-fermentable carbon sources, but no phenotypic differences between the different CAT mutants could be observed, indicating that the effect is a compensation for the peroxisomal citrate synthase and does not improve growth in CAT-gene specific manners. This set of data suggests that the main role of the glyoxylate cycle is indeed the synthesis of C_4 intermediates. The enhanced growth of the Δ*cit2* single mutant compared to the Δ*cit2*Δcat double mutants in the presence of carnitine furthermore indicates that complementation through a functional carnitine shuttle is more effective than supplying the cell with C_4 intermediates.

Our data strongly suggest that carnitine plays other, previously unrecognised roles in *S. cerevisiae*. The compound has a clear growth enhancing impact on wild type strains growing on malate and ethanol containing medium, as well as on plates containing

inhibiting concentrations of lactate. The impact of carnitine in these conditions appears entirely independent of the three CAT enzymes and the carnitine shuttle, since the enhancement is observed in the Δcit2Δcat double mutants that express the malate transporter *MAE1* and in the triple CAT deletion strain when grown in the presence of inhibitory concentrations of lactate. These data strongly suggest a role for carnitine in alleviating the toxic effects of certain organic acids.

Acetic acid toxicity appeared not to be affected by carnitine. This may be explained by the finding that lactate and acetate inhibit yeast growth in different manners, with acetate leading to an increase in cellular ATP demand required for maintenance, while lactate appears to affect cells in a different and unknown manner (Maiorella *et al*. 1983; Narendranath *et al*. 2001). The underlying mechanisms of this phenotype need further investigation and will be the focus of future studies.

The investigation of other stress conditions did not reveal any impact of carnitine on NaCl induced osmotic stress tolerance. This observation can be linked to reports that carnitine uptake via the membrane transporter Agp2 is shut down by the Hog1p MAP kinase pathway when exposed to high concentrations of sodium chloride (Lee *et al*. 2002).

The data also show that the compound appears not to increase cellular survival during heat or oxidative shocks. No effect was observed when growing cells in the presence or absence of carnitine before administration of such shock. However, the presence of carnitine clearly enhances growth when cells are grown in stressful conditions. Indeed, carnitine that was added to YND plates containing H_2O_2 clearly enhanced the yeast's ability to grow under these conditions. This effect was dependent on the transcriptional activator Yap1p, which regulates several enzymes which protects against oxidants (Grey and Brendel 1994; Hussain and Lenard 1991; Schnell *et al*. 1992), suggesting some direct molecular role for carnitine in the process.

Further studies are required to evaluate the effect of carnitine addition in cells grown under oxidative stress conditions on cellular oxidation levels. It will also be of interest to establish if the protection offered by carnitine against hydrogen peroxide is related to that observed for the organic acids, malate and lactate, since it is known that the presence of organic acids can result in oxidative stress (for review see Piper *et al*. 2001).

The results indicate that deletion of *CAT2* significantly decreases survival of the mutant strain when exposed to either oxidative stress or pre-adaptive thermal stress

(FY23Δ*cat2* survival vs other strains, p < 0.05). Since the cultures used for the experiments were grown in media containing a non-fermentable carbon source (YNG), to ensure that the enzymes involved in the carnitine shuttle are actively expressed, resistance to such stresses are intrinsically higher due to the activities associated with respiratory growth (Jamieson 1992; Barros *et al*. 2004). A decrease in survival does therefore not necessarily indicate a decrease in the efficiency of the yeasts defence systems, but possibly an indirect effect on peroxisomal or mitochondrial function caused by the deletion of *CAT2*. The inability of the Δ*cat2* mutant strains to respond to pre-adaptation could be a result of the associated build-up of reactive oxygen species in these conditions. It is intriguing that this effect is independent of carnitine and therefore also of the carnitine acetyl transferase activity of Cat2p. This *CAT2* effect was also not apparent when the strains were tested for growth in the presence of H_2O_2, where *CAT2* gene deletion did not lead to any significant change in phenotype (results not shown). However, these experiments can only be carried out on fermentable carbon sources, and it is therefore possible that the CAT enzymes are either not expressed or not functional in these conditions. No significant effect on survival was observed for the other two CATs, Yat1p and Yat2p. It will, however, be necessary to investigate the effect of CAT deletion and overexpression on cellular oxidation status to gain a clearer insight into the effects of these enzymes on oxidative stress.

Several lines of evidence indicate that carnitine and the shuttle have pleiotropic and beneficial effects in higher eukaryotic cells (Steiber *et al*. 2004). Furthermore, mutations in higher eukaryotic acyltransferases can give rise to severe metabolic disorders (for review see Ramsay and Zammit 2004). Many of these impacts have been attributed to the role of the shuttle in energy metabolism, the balancing effect on compartmentalised pools of CoA in the cell and the removal of harmful organic acids through transfer to carnitine. However, carnitine has recently been implicated in enhancing stress resistance by up-regulating certain elements of the mammalian heat shock response, thereby creating a proposed cytoprotective state (Calabrese *et al*. 2006). These findings, together with carnitine's potential as an anti-oxidant and the increasing research focus on a role for carnitine as a therapeutic agent with several possible applications, highlight the potential importance of the phenotypes described in this paper. Indeed, the investigation of the molecular nature of carnitine-dependent but shuttle-independent phenotypes will be easier in an accessible model system such as *S. cerevisiae*.

3.5. REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G. (1994). Current Protocols in Molecular Biology. John Wiley & Sons, New York

Barros, M.H., Bandy, B., Tahara, E.B., Kowaltowski, A.J. (2004). Higher respiratory activity decreases mitochondrial reactive oxygen release and increases lifespan in *Saccharomyces cerevisiae*. *J Biol Chem* **279,** 49883-49888

Berben, G., Dumont, J., Gilliquet, V., Bolle, P.A., Hilger, F. (1991). The YDp plasmids: a uniform set of vectors bearing versatile gene disruption cassettes for *Saccharomyces cerevisiae*. *Yeast* **7,** 475-477

Bieber, L.L. (1988). Carnitine. *Annual review of biochemistry* **57,** 261-283

Calabrese, V., Giuffrida Stella, A.M., Calvani, M., Butterfield, D.A. (2006). Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes. *J Nutr Biochem* **17,** 73-88

Gietz, R.D., Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74,** 527-534

Grey, M., Brendel, M. (1994). Overexpression of the *SNQ3/YAP1* gene confers hyperresistance to nitrosoguanidine in *Saccharomyces cerevisiae* via aglutathione-independent mechanism. *Curr Genet* **25,** 469-471

Hussain, M., Lenard, J. (1991). Characterisation of *PDR4*, a *Saccharomyces cerevisiae* gene that confers pleiotropic drug resistance in high copy number. *Gene* **101,** 149-152

Jamieson, D.J. (1992) *Saccharomyces cerevisiae* has distinct responses to both hydrogen peroxide and menadione. *J Bacteriol* **174,** 6678-6681

Kawahata, M., Masaki, K., Fujii, T., Iefuji, H. (2006). Yeast genes involved in response to lactic and acetic: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. *FEMS Yeast Res* **6,** 924-936

Kispal, G., Sumegi, B., Dietmeier, K., Bock, I., Gajdos, G., Tomcsanyi, T., Sandor, A. (1993). Cloning and sequencing of a cDNA encoding *Saccharomyces cerevisiae* carnitine acetyltransferase. Use of the cDNA in gene disruption studies. *J Biol Chem* **268,** 1824-1829

Kunau, W.H., Dommes, V., Schulz, H. (1995). Beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. *Prog Lipid Res* **34,** 267-342

Lee, J., Lee, B., Shin, D., Kwak, S., Bahk, J.D., Lim, C.O., Yun, D. (2002). Carnitine uptake by *AGP2* in yeast *Saccharomyces cerevisiae* is dependent on Hog1 MAP kinase pathway. *Moll Cells* **13,** 407-412

Leenders, F., Tesdorpf, J.G., Markus, M., Engel, T., Seedorf, U., Adamski, J. (1996). Porcine 80-kDa protein reveals intrinsic 17 beta-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer activities. *J Biol Chem* **271,** 5438-5442

Maiorella, B., Blanch, H.W., Wilke, C.R. (1983) By-product inhibition effects on ethanolic fermentation *Saccharomyces cerevisiae*. *Biotech Bioeng* **25,** 103-121

Narendranath, N.V., Thomas, K.C., Ingledew, W.M. (2001). Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in minimal medium. *J Ind Microbiol Biotechnol* **26,** 171-177

Palmieri, L., Lasorsa, F.M., De-Palma, A., Palmieri, F., Runswick, M.J., Walker, J.E. (1997). Identification of the yeast *ACR1* gene product as a succinate-fumarate transporter essential for growth on ethanol or acetate. *FEBS letters* **417,** 114-118

Piper, P., Calderon, C.O., Hatzixanthis, K., Mollapour, M. (2001). Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* **147,** 2635-2642

Ramsey, R.R., Zammit, V.A. (2004) Carnitine acetyltransferases and their influence on CoA pools and disease. *Mol Aspects Med* **25,** 475-493

Reddy, J.K., Mannaerts, G.P. (1994) Peroxisomal lipid metabolism. *Annu Rev Nutr* **14,** 343-370

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schmalix, W., Bandlow, W. (1993). The ethanol-inducible *YAT1* gene from yeast encodes a presumptive mitochondrial outer carnitine acetyltransferase. *J Biol Chem* **268,** 27428-27439

Schnell, N., Krems, B., Entian, K.D. (1992). The *PAR1*(*YAP1/SNQ3*) gene of *Saccharomyces cerevisiae*, a c-*Jun* homologue, is involved in oxygen metabolism. *Curr Genet* **21,** 269-273

Schulz, H. (1991). Beta oxidation of fatty acids. *Biochimica et biophysica acta* **1081,** 109-120

Steiber, A., Kerner, J., Hoppel, C.L. (2004). Carnitine: a nutritional, biosynthetic and functional perspective. *Mol Aspects Med* **25,** 455-473

Stemple, C.J., Davis, M.A., Hynes, M.J. (1998). The *facC* gene of *Aspergillus nidulans* encodes an acetate-inducible carnitine acetyltransferase. *J Bacteriol* **180,** 6242-6251

Swiegers, J.H., Dippenaar, N., Pretorius, I.S., Bauer, F.F. (2001). Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: three carnitine acetyltransferases are essential in a carnitine-dependent strain. *Yeast* **18,** 585-595

Van Roermund, C.W., Elgersma, Y., Singh, N., Wanders, R.J., Tabak, H.F. (1995). The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. *EMBO* **14,** 3480-3486

Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F., Subden, R.E., Young, R.A., Lonvaud, A., Denayrolles, M., van-Vuuren, H.J. (1997). Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. *Nat Biotechnol* **15,** 253-257

Wanders, R.J., Schutgens, R.B., Barth, P.G. (1995). Peroxisomal disorders: a review. *J Neuropathol Exp Neurol* **54,** 726-739

Westermann, B., Neupert, W. (2000). Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in *Saccharomyces cerevisiae*. *Yeast* **16,** 1421-1427

Winston, F., Dollard, C., Ricupero-Hovasse, S.L. (1995). Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* **11,** 53-55

Chapter 4

RESEARCH RESULTS II

General regulators of the oxidative stress response and cytochrome c are required for protective and detrimental effects of L-carnitine in *Saccharomyces cerevisiae*.

ABSTRACT

L-Carnitine plays a well documented role in eukaryotic energy homeostasis by acting as a shuttling molecule for activated acyl residues across intracellular membranes. This activity is supported by carnitine acyl-transferases and transporters, and is referred to as the carnitine shuttle. However, several pleiotropic and often beneficial effects of carnitine in humans have been reported that appear to be unrelated to the shuttling activity, but little conclusive evidence regarding the molecular networks that would be affected by carnitine exist. We have recently demonstrated a protective role of carnitine in cellular stress in yeast that is independent of the carnitine shuttle. Here we show that carnitine specifically protects against oxidative stress caused by hydrogen peroxide and the superoxide generating agent menadione. The data also indicate that carnitine has a detrimental effect on cellular survival when combined with thiol-modifying agents. A genetic analysis indicates that central elements of the oxidative stress response, in particular the transcription factors Yap1p and Skn7p, are required for carnitine to exert its protective effect, but that several downstream effectors of the response are dispensable. A DNA microarray-based global gene expression analysis identified Cyc3p, a cytochrome c heme lyase, as being important for carnitine's protective impact in oxidative stress conditions. These findings establish a direct genetic link to a carnitine-related phenotype that is independent of the shuttle system. The data suggest that the yeast *Saccharomyces cerevisiae* should provide a useful model for further elucidation of carnitine's physiological roles.

4.1. INTRODUCTION

The role of L-carnitine in the carnitine shuttle has been well characterized for its function in the transfer of the activated products of energy metabolism between intra-cellular compartments (Bieber, 1988; Reddy and Manaerts 1994). Activated acyl residues are transferred to carnitine by the activities of various carnitine acyl transferases and transported by carnitine/acylcarnitine carriers across the membranes of the mitochondria and peroxisomes. Collectively, this molecular network is referred to as the carnitine shuttle. The carnitine shuttle of *Saccharomyces cerevisiae* closely resembles that of higher eukaryotes in function, with minor differences in composition. Most notably, yeast appears to only posses carnitine acetyl-transferase activity, in contrast to the long chain acyl-transferase activities present in higher eukaryotes (Kispal *et al*, 1993). In yeast, this activity is ascribed to three carnitine acetyl-transferases (CATs), Yat1p, Yat2p and Cat2p, compared to a single mitochondrial CAT in mammalian systems (Schmalix an Bandlow, 1993; Swiegers *et al*, 2001). Furthermore, in yeast the glyoxylate cycle provides an alternative route by which activated acetyl-residues can gain access to the mitochondrial tricarboxilic acid cycle (Plamieri *et al*, 1997; Van Roermund *et al*, 1995). A final difference between yeast and mammals is that in yeast acetyl coenzyme A is generated both in cytosol and peroxisomes, compared to the exclusive peroxisomal acetyl-CoA generation in higher eukaryotes (Kunau *et al*, 1995).

Carnitine supplementation results in various beneficial effects in human subjects or cell lines. Most notably supplementation of carnitine and of various acylcarnitines has been associated with protecting against neurodegeneration and mitochondrial decay resulting from ageing and against the onset of apoptosis in various cell lines (Calabrese *et al*, 2005, Calabrese *et al*, 2006, Rani and Panneerselvam, 2001), also suggesting a link to oxidative stress protection. Several hypotheses have been generated to explain these effects. These include the upregulation of the mammalian stress responsive gene HO-1, encoding a heme oxygenase (Calabrese *et al*, 2006; Calo *et al*, 2006). Furthermore, carnitine has also been postulated to have a possible intrinsic antioxidant capacity which has been proposed to be involved in some of the observed effects in neuronal apoptosis protection (Gulcin, 2006; Silva-Adaya *et al*, 2008). Carnitine, through its involvement in energy metabolism, has been reported to have a stimulatory effect on mitochondrial metabolism, which has been linked to the benefits of carnitine

and acetylcarnitine supplementation. In particular, feeding carnitine to rats was shown to reverse the age dependent decline in mitochondrial membrane potential and decline in cardiolipin levels (Hagen *et al*, 1998). In thioredoxin deficient DT 40 cells acetylcarnitine was shown to specifically protect against oxidative stress in and around mitochondria, including the release of cytochrome c and *SOD1* (Zhu *et al*, 2008). However, it is unclear if these changes are influenced directly or indirectly by the presence of carnitine and acylcarnitines. Moreover, no direct mechanisms have thus far been proposed to explain the different, generally beneficial effects of dietary carnitine supplementation.

Recently we have demonstrated a role for carnitine in the protection against weak organic acid stress induced by for example acetate and oxidative stress induced by hydrogen peroxide in the eukaryotic model organism, *Saccharomyces cerevisiae* (Franken *et al*, 2008). This effect was found to be independent of all yeast CATs and therefore also of the carnitine shuttle. Here we present an analysis of the protective effect of carnitine against oxidative stress in yeast. The data indicate that carnitine specifically protects against the effects of the ROS generating agents H_2O_2 and menadione. Intriguingly, carnitine has an opposite and detrimental effect when combined with the thiol modifying agents diamide and DTT. This observation is the first report of a damaging effect associated with carnitine supplementation. The data indicate that carnitine has no free radical scavenging activity. A genetic analysis reveals that carnitine requires genetic mediation, in particular the general regulators of the oxidative stress response, encoded by *YAP1* and *SKN7*, to exert its effect. A global gene expression analysis by DNA microarray comparing stressed and unstressed yeast grown in the presence and absence of carnitine led to the identification of the cytochrome c heme lysase Cyc3p as being important for carnitine-mediated stress protection. This finding links carnitine to mitochondrial functions, and suggests several possible pathways through which carnitine may exert its molecular effects.

4.2. EXPERIMENTAL PROCEDURES

4.2.1. YEAST STRAINS AND MEDIA

The wild type strain BY4742 and all single gene knock-outs used in this study were obtained from Euroscarf (Frankfurt, Germany). The strains in which Yap1p and Msn2p have been replaced with GFP tagged versions of the same proteins were purchased

from Invitrogen. All strains are derived from the S288c genetic background and were grown either on YPD or minimal YND media containing 0.67% (w/v) yeast nitrogen base (YNB) without amino acids (DIFCO) and 2% (w/v) glucose supplemented with amino acids according to the specific requirements of the respective strains.

4.2.2. MULTI-COPY EXPRESSION OF *CYC3* AND CREATION OF THE ΔCYC1ΔCYC7 DOUBLE MUTANT

The plasmids and constructs used in this study are listed in Table 4.1 and the primers for the amplification of *CYC3* and also the *CYC1* integration cassette are listed in Table 4.2. Standard DNA techniques were used for the isolation and manipulation of DNA throughout the study (Sambrook *et al*, 1989; Ausubel *et al*, 1994). Restriction enzymes, T4 DNA-ligase and Expand Hi-Fidelity polymerase used in the enzymatic manipulation of DNA were obtained from Roche Diagnostics (Randburg, South Africa) and used according to the specifications of the supplier. Escherichia coli DH5α (GIBCO-BRL/Life Technologies) was used as host for the construction and propagation of all plasmids. Sequencing of all plasmids was carried out on an ABI PRISMTM automated sequencer.

TABLE 4.1. Plasmids and constructs used in this study

Plasmids	Relevant genotype	Sources and references
pGEM-T-easy		Promega
pGEM-T-easy-*CYC3*		This study
YEplac195	*2μ URA3*	Gietz and Sugino, 1988
YEplac195-CYC3	*2μ URA3 CYC3*	This study
YDp-U	*2μ URA3*	Berben *et al*, 1991

For the cloning of *CYC3*, the primer pair CYC3-F and CYC3-R was used, which amplifies a 1551 bp fragment containing the *CYC3* promoter, open reading frame (ORF) and terminator. The amplified fragment was ligated into the cloning vector pGEM-T-easy (Promega). The *CYC3* gene cassette was excised using the *Eco*RI site from the pGEM-Teasy vector and a *Sal*I site upstream of the *CYC3* STOP codon. The excised fragment was ligated into the plasmid YEpLac195 (Gietz and Sugino, 1988) using *Eco*RI and *Sal*I restriction sites.

For the amplification of the *CYC1* disruption cassette the primer pair CYC1-URA3int-Fp and CYC1-URA3int-Rp was used, which amplifies the *URA3* cassette from the plasmid YDp-U and incorporates 5' and 3' flanking regions homologues to regions outside the *CYC1* ORF. The amplified disruption cassette was transformed into the BY4742Δ*cyc7* strain to create the Δ*cyc1*Δ*cyc7* double mutant. Integration was verified by PCR using the primers CYC1-F and CYC7-R.

Table 4.2. Primers used in this study. Sequences with homolgy to the *URA3* region of the plasmid YDp-U are underlined

Primer	Sequence
CYC3-F	5-'GGAGCAAGTTGTGGTTTACAACACC -3'
CYC3-R	5'-CGAGACGAATGGCGACATTTG-3'
CYC1-URA3int-Fp	5'-ATGTGTGCGACGACACATGATCATATGGCATGCATGTGCTCTGT<u>GCTGCAGGTCGACGGATCCG</u>-3'
CYC1-URA3int-Rp	5'-GTGGGAGGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATG<u>GGTGATTGATTGAGCAAGCTGG</u>-3'
CYC1-F	5'-GCAAGATCAAGATGTTTTCACCG-3'
CYC1-R	5'-ATAATGTTACATGCGTACACGCG-3'

4.2.3. ABTS ANTIOXIDANT ASSAY

To determine the antioxidant capacity of carnitine the ABTS [2,2'-azinobis-(-3-ethyl-benzothiazolin-6-sulfonic acid)] radical cation decolouration assay was used as previously described (Re *et al*, 1999; De Beer *et al*, 2003). The assay can be effectively used for lipophilic and hydrophilic antioxidants and is based on the preformation of the blue/green ABTS•+ chromophore by oxidation with potassium persulfate, which is then reduced in the presence of hydrogen donating antioxidants. The resulting decolouration can subsequently be measured spectrophotometrically. The ABTS•+ radical monocation was formed by addition of 88 µl of a 140 mM potassium persulfate solution to 5 ml of 7 mM ABTS, which was protected from light and incubated overnight at room temperature. The ABTS solution was diluted to and O.D. 0.7 at 734 nm for use in the assay. Dilution series of 50, 100, 150, 200, 300 and 400 mM were set up for Trolox, ascorbic acid, γ-butyrobetaïne and L-carnitine. The reaction mixture consisted of 200 µl of the diluted ABTS solution to which 10 µl of each of the samples from the mentioned dilution series was added. ABTS decolouration was measured after 4 min at O.D. of 734nm. Assays were done in triplicate.

4.2.4. PREPERATION OF PLATES CONTAINING REDOX STRESS INDUCING AGENTS

The plates used for the assessment of the effect of various oxidants (H_2O_2, menadione, linoleic acid hydroperoxide (LoaOOH), cumene hydroperoxide (CHP) and diamide) and also the thiol reducing agent DTT in combination with carnitine were prepared on the day before cultures were spotted. SCD agar medium was left to cool to 50°C before the addition of the oxidants (0.5 and 1.5 mM H_2O_2; 0.1 and 0.2 mM menadione; 1.0 and 1.5 mM LoaOOH; 0.1 and 0.25 CHP; 0.8 and 1.2 mM Diamide; 8.0 and 16 mM DTT) and also L-carnitine (added in a concentration range between 50 and 1500 mgL^{-1}). Plates were stored overnight at 4°C in the dark. Linoleic acid hydroperoxide was prepared as described by Evans and co-workers (Evans *et al*, 1998).

4.2.5. DETERMINATION OF INTRACELLULAR ROS

The oxidant sensitive probe 2',7'-dichlorofluoresciein diacetate (DCFH-DA, Sigma) was used for the detection of intracellular ROS. The dye is cell permeable and widely used for the rapid quantification of ROS in eukaryotic cells (Jakubowski and Bartosz, 1997). The DCFH-DA probe is nonfluorescent until acetate groups are removed by intracellular esterases and can then be oxidized by intracellular ROS to the fluorescent compound 2',7'-dichlorofluorescein (DCF), which can be detected as a indirect measure of intracellular ROS. Cultures of BY4742 were grown overnight to inoculate fresh SCD and media and also SCD containing carnitine (1000 mgL^{-1}) to an O.D.$_{600}$ of 0.1. A duplicate set was also inoculated, which was to be treated with 0.6 mM H_2O_2 for 30 and 90 min respectively, when the cultures reached mid-log phase. After treatment the cultures were harvested, washed once and resuspended in phosphate buffered saline (PBS). Cells were diluted to ~ 10^6 per ml; DCFH-DH was added to a concentration of 100 μM and incubated for 30 min at 28 °C. Data were acquired using the BD FACSAria cell sorter, equipped with 407 nm, 488 nm and 633 nm lasers and the BD FACSDiva 6.1 software. The samples were acquired with an event rate of 600 per second using a 70 μm nozzle. Intensity histogram overlays were generated using FlowJo 2.1.1. The experiment was done in triplicate.

4.2.6. MICROARRAY ANALYSIS

Cultures of the wild type strain BY4742 was grown overnight in SCD containing tubes to serve as pre-cultures. Two flasks containing 50ml of freshly prepared SCD media and also two containing SCD with carnitine (1000 mgL^{-1}) was inoculated to an $O.D._{600}$ ~ 0.1 and grown to mid-log phase ($O.D._{600}$ 0.4 -.0.5). A duplicate set was also inoculated and exposed to 0.4 mM H_2O_2 for 30 min before harvesting and isolation of total RNA. RNA was isolated as previously described (Schmitt *et al*, 1990). Probe preparation and hybridization to Affymetrix Genechip® microarrays were performed according to Affymetrix instructions, starting with 6 µg of total RNA. Results for each condition were derived from 2 independent culture replicates. The quality of total RNA, cDNA, cRNA and fragmented cRNA were confirmed using the Agilent Bioanalyzer 2100.

4.2.7. TRANSCRIPTOMICS DATA ACQUISITION AND STATISTICAL ANALYSIS

Acquisition and quantification of array images and data filtering were performed using Affymetrix GeneChip® Operating Software (GCOS) version 1.4. All arrays were scaled to a target value of 500 using the average signal from all gene features using GCOS. Genes with expression values below 12 were set to 12+ the expression value as previously described (Boer *et al*, 2003) in order to eliminate insignificant variations. Determination of differential gene expression between experimental parameters was conducted using SAM (Significance Analysis of Microarray) version 2. The two-class, unpaired setting was used and genes with a Q value of 0.5 ($p < 0,005$) were considered differentially expressed. Only genes with a fold change greater than 1.8 (positive or negative) were taken into consideration.

4.3. RESULTS

4.3.1. RELATIONSHIP OF L-CARNITINE TO KNOWN REDOX STRESSORS

It has previously been established that carnitine supplementation enhances growth of yeast strains in conditions of organic acid stress and also oxidative stress induced by hydrogen peroxide (Franken *et al*, 2008). Since a range of oxidative stress inducing agents have been identified and their effects on the physiology of yeast cultures have been reasonably well characterized (Thorpe *et al*, 2004; Gasch *et al*, 2000), it was of interest to investigate the effect of carnitine supplementation of cultures exposed to these compounds. The compounds that were chosen for this study include the ROS

generating oxidants hydrogen peroxide, the superoxide generating agent menadione, linoleic acid hydroperoxide (LoaOOH, a byproduct of lipid peroxidation), cumene hydroperoxide (CHP, an aromatic hydroperoxide) and also the thiol oxidizing agent diamide.

With regards to ROS generating oxidants, carnitine visibly enhances growth of exposed cultures in all cases except for exposure to CHP (Figure 4.1 A). Although there is a growth enhancing effect in the case of linoleic acid hydroperoxide, it is not as clear as in the case of H_2O_2 and menadione.
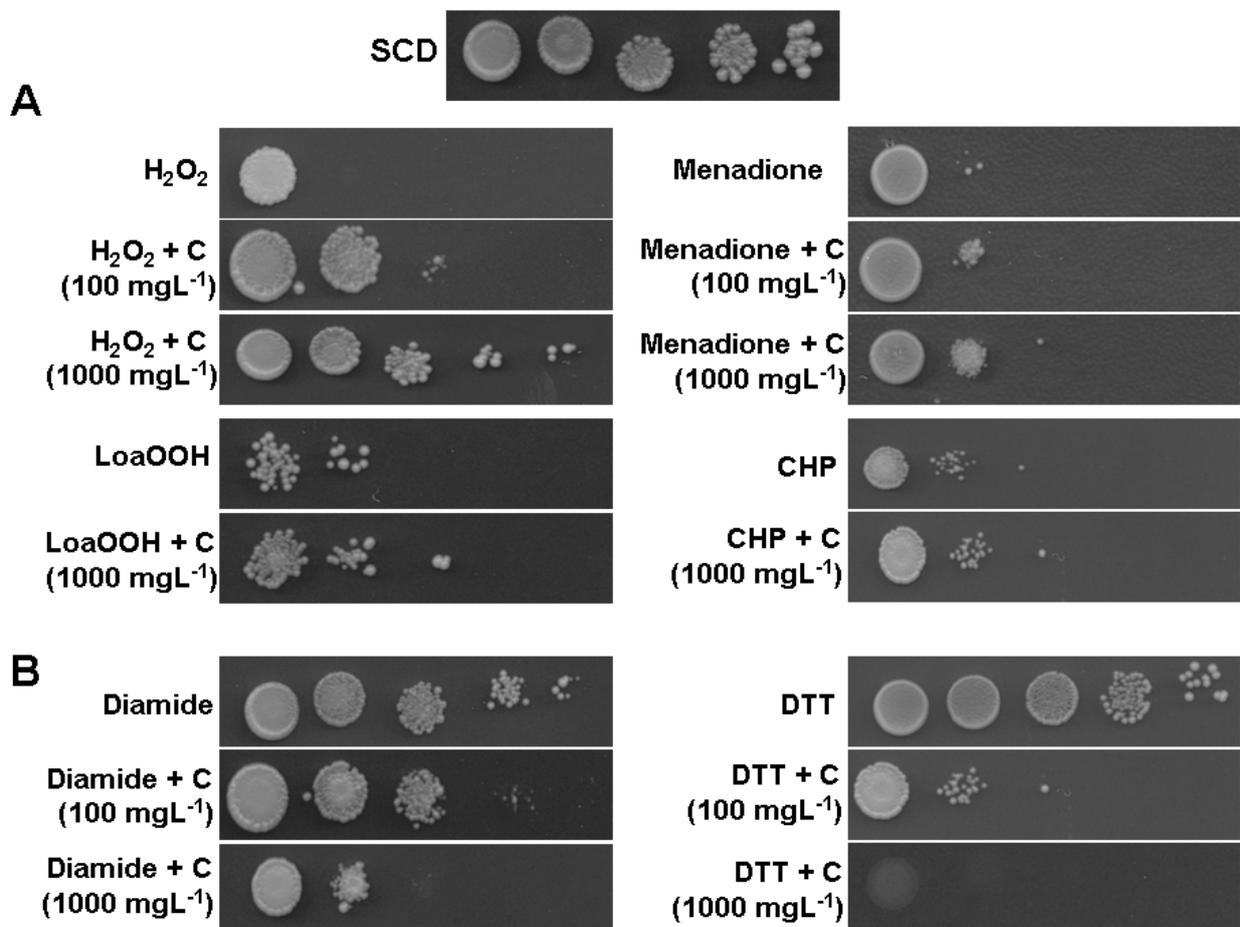
Figure 4.1. Carnitine supplementation (100 and 1000 mgL^{-1}) in combination with redox stress inducing agents. (**A**) ROS inducing stressors: H_2O_2 (1.5 mM), Menadione (0.1 mM), LoaOOH (1.0 mM) and CHP (0.8 mM). (**B**) Thiol modifying agents: the thiol oxidizing agent diamide and the thiol reducing agent DTT was used at final concentrations of 1.2 mM and 8 mM respectively.

Interestingly carnitine addition in combination with the thiol oxidant diamide leads to a significant decrease in growth compared to cultures that are only exposed to the oxidant (Figure 4.1 B). Similar results are observed in combination with the thiol reducing agent dithiothrietol (DTT). The effect of carnitine is concentration dependent,

with effects on plates being visible from below 100 mgL^{-1} upwards. This effect was observed in the case of protection against H$_2$O$_2$ and menadione and also for the damaging effects of diamide and DTT (Figure 4.1). Furthermore, when liquid (SCD) cultures were grown to mid-log phase in the presence of carnitine, harvested, washed and spotted on peroxide plates (without carnitine supplementation), carnitine was found to excert a protective effect at concentrations of 1 mgL^{-1} in the liquid medium (data not shown).
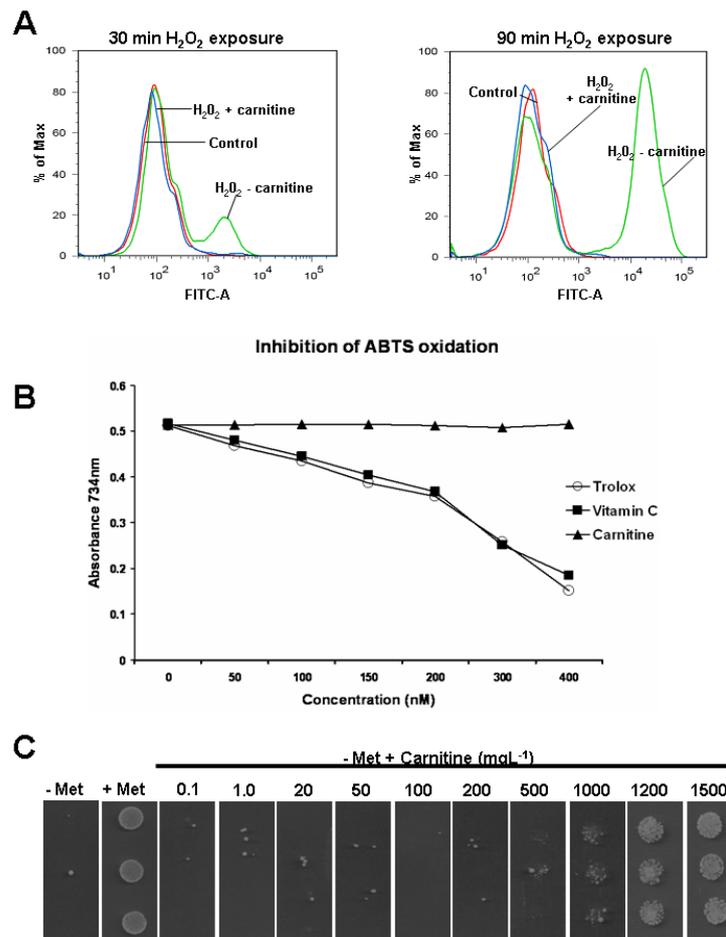
Figure 4.2. Assessment of carnitine's possible role as an antioxidant. (**A**) Flow-cytometer profiles of ROS accumulation in strains treated with 0.6 mM H$_2$O$_2$ for 30 and 90 min with and without carnitine supplementation detected by the fluorescent probe DCFH-DA. (**B**) Carnitine does not exhibit the ability to scavenge free radicals using the ABTS decoloration assay, ascorbate and Trolox was used as controls. (**C**), Carnitine supplementation rescues the synthetic methione auxotrophy of a Δ*sod2* strain under aerobic growth conditions in a concentration dependent manner.

4.3.2. CARNITINE DOES NOT SCAVENGE FREE RADICALS, BUT BEHAVES LIKE AN ANTIOXIDANT IN A BIOLOGICAL CONTEXT

A role for carnitine and some of its esters have been clearly indicated in protecting against the onset of apoptosis, ageing and other states associated with the build-up of

oxidative stress (Rani and Panneerselvam, 2001). As indicated, carnitine addition to yeast cultures exposed to 0.6 mM H_2O_2 was, similar to observations in higher organisms, able to dramatically decrease the amount of intracellular ROS in yeast, as detected by the fluorescent probe DCFH-DA (Figure 4.2 A). One line of argumentation suggests that this could be due the antioxidant effect of carnitine itself (Gulcin, 2006). To investigate if L-carnitine is indeed able to scavenge free radicals, the molecule was used in an ABTS free radical scavenging assay. As is clear in Figure 4.2 B, carnitine is unable to scavenge free radicals and would, therefore, be unable to function as a molecular antioxidant.

Using carnitine in a yeast based biological antioxidant screen proposed by Žyracka and co-workers (Žyracka *et al*, 2005; Figure 4.2 C) illustrates that carnitine addition to cultures of a Δ*sod1* strain abolishes the synthetic methionine auxotrophy of the strain under aerobic conditions. The effect of carnitine is concentration dependent and comparable to the effects reported for antioxidants such as ascorbate, glutathione, cystein and N-acetylcystein (Žyracka *et al*, 2005). γ-Butyrobetaïne, the direct precursor of carnitine in the eukaryotic carnitine biosynthesis pathway and differing only by a hydroxyl group on the third carbon from carnitine, did not show any free redical scavenging activity and was unable to complement the auxotrophy of Δ*sod1*.

4.3.3. THE PROTECTIVE EFFECT OF CARNITINE REQUIRES THE MAJOR PATHWAYS INVOLVED IN OXIDATIVE STRESS PROTECTION

In yeast, the main responses to oxidative stress are regulated by the transcription factors Yap1p, Msn2p and also Skn7p (Jamieson, 1998). These responses include the upregulation of antioxidant enzymes, such as the superoxide dismutases, *SOD1* and *SOD2*, and enzymes involved in glutathione metabolism, such as *GLR1*. To assess if the action of carnitine requires these pathways, strains bearing deletions of genes involved in these pathways were assessed for their oxidative stress response in the presence or absence of carnitine. The genes assessed included *YAP1* and genes encoding regulators of its activity, including the Yap1p binding protein Ybp1 (Veal *et al*, 2003) and the thiol peroxidase Hyr1p (Delaunay *et al*, 2002), which transduces a redox signal to Yap1p, as well as the transcription factor Skn7p and the downstream antioxidant enzymes Sod2p (superoxide dismutase, Saffi *et al*, 2006), Tsa1p (a thioredoxin peroxidase, Chae *et al*, 1994), Trx2p (a thioredoxin, Pedrajas *et al*, 1999), Glr1p (a glutathione oxidureductase, Grant, 2001) and Gsh1p (catalyzes the first step in

glutathione biosynthesis, Ohtaki and Yabuuchi, 1991). The strains were spotted on plates containing H_2O_2 (0.5 and 1.0 mM) with and without the addition of carnitine to a final concentration of 1000 mgL^{-1}. From the results (Figure 4.3) it is clear that carnitine supplementation leads to enhanced survival in the case of Δ*sod2*, Δ*tsa1* and Δ*trx2* strains. In the case of gene deletion of *YAP1*, *SKN7* and the genes required for glutathione metabolism, carnitine was unable to compensate for the deletion of the respective genes.

It has been indicated that certain molecules, such as green tea polyphenols, are able to induce the activation of Yap1p and also Msn2p, localized in the cytosol under non-stress conditions, which leads to the nuclear localization of the two transcription factors in order to activate target gene sets (Maeta *et al*, 2007). Considering that carnitine has been proposed to act through the mammalian regulator of stress induced genes, namely Nrf2 (Calabrese *et al*, 2005), the effect of carnitine on the localization of Yap1p and Msn2p using GFP-fusions of the two transcription factors was investigated. Cultures exposed to carnitine did, however, not lead to a change in localization of either Yap1p or Msn2p (data not shown).
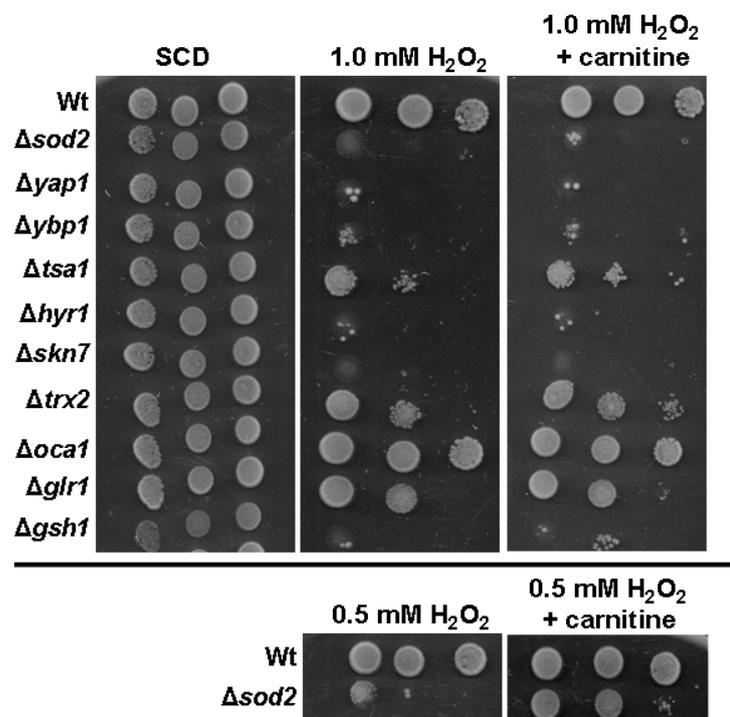
Figure 4.3. The effect of carnitine supplementation on strains with deletions of genes required for the cells defense against oxidative stress. The transcription factors Yap1p and Skn7p and genes involved in glutathione metabolism (*GSH1* and *GLR1*) are required for carnitine's protective effect, whereas carnitine supplementation results in enhanced growth of strains with deletions of the antioxidant enzymes *SOD2*, *TRX2* and *TSA1*.

4.3.4. SCREENING FOR POSSIBLE GENETIC LINKS TO THE PROTECTION AGAINST OXIDATIVE STRESS BY CARNITINE

To screen for possible genetic mediators of the protective effect of carnitine, microarray analysis was performed, comparing the wild type strain BY4742 grown in SCD with and without carnitine supplementation. In addition global expression analysis of cultures grown to mid-log (with and without carnitine addition) and then exposed to H_2O_2 was also performed. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE16346 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE16346).

Table 4.3. Genes upregulated by carnitine and genes of overrepresented transcription factors screened on H_2O_2 and H_2O_2 with carnitine (1000 mgL^{-1}). Only deletion of *CYC3*, showed decreased responsiveness to carnitine. Two strains, highlighted in shaded blocks, were included because the genes showed significant up-regulation in the microarrays comparing yeast cells in the presence or absence of carnitine without H_2O_2.

Deletion strains with WT-level H_2O_2 sensitivity (expression fold change: H_2O_2 + carnitine *vs* H_2O_2 – carnitine)	Deletion strains with above WT H_2O_2 sensitivity (expression fold change: H_2O_2 + carnitine *vs* H_2O_2 – carnitine)
Deletion mutants of overrepresented transcription factors	
Δ*aft2* (-1.48); Δ*cad1* (-1.08)	Δ*aft1* (1.07); Δ*sok2* (-1.32); Δ*rpn4* (-1.21)
Strains deleted for genes with up-regulated transcripts (fold-change)	
Δ*uip4* (2.01); Δ*alp1* (2.01); Δ*jlp1* (2.03); Δ*gut2* (2.09); Δ*gdb1* (2.19); Δ*hpf1* (1.97); Δ*lee1* (2.27); Δ*cos111* (2.33); Δ*tkl2* (3.44); Δ*gnd2* (3.77); Δ*sps100* (3.18); Δ*gph1* (1.97); Δ*cox23* (1.94); Δ*ald3* (1.86); Δ*gsy1* (1.80); Δ*hes1* (1.43); Δ*adr1* (1.49); Δ*mig2* (1.85); Δ*ras1* (1.86); Δ*ykr075*c (2.56); Δ*ydl218*w (2.02); Δ*ykl187*c (2.33); Δ*yfl052*w (2.71); Δ*rtn2* (1.93)	Δ*ubr2* (2.01); Δ*sip18* (2.14); Δ*ptr2* (2.25); Δ*hbt1* (2.43); Δ*mth1* (2.52); Δ*put4* (2.61); Δ*tsa2* (1.05); Δ*pai3* (1.97); Δ*nca3* (1.94); Δ*yor1* (1.81); Δ*ybr285*w (2.03); Δ*ypl113*c (2.50); Δ*yol131*w (2.25); Δ*cyc3* (1.99)

Using a fold cut-off value of 1.8, 40 genes were found to be upregulated and also 40 downregulated when comparing the peroxide treated cultures supplemented with carnitine to the unsupplemented, peroxide treated set. Comparing the carnitine treated

to untreated sets revealed only 5 genes up and 12 genes downregulated. Deletion mutants representing all of the identified, non-essential, genes upregulated by the presence of carnitine during peroxide stress were spotted in serial dilutions on plates containing 0.75 mM and 1.6 mM H_2O_2 and also on a duplicate set which contained carnitine (added to 1000 mgL^{-1}). Also included in the set of mutants were strains bearing deletions of *AFT1*, *AFT2*, *CAD1*, *RPN4* and *SOK2*, which were identified as overrepresented transcription factors within the set of upregulated genes using the yeast transcription factor database, Yeastract (http://www.yeastract.com/). In addition, strains bearing deletions of *HES1* and *TSA2*, were included, since both genes were upregulated by the presence of carnitine in non-stressed conditions. The plates were incubated at 30°C for three days and inspected for growth. As indicated in Table 4.3, 14 out of the 38 upregulated genes that were screened were sensitive to H_2O_2 present in the media. Out of the 5 overrepresented transcription factors, Δ*aft1*, Δ*sok2*, Δ*rpn4* also tested sensitive to peroxide. However, only in one case, Δ*cyc3*, could a decrease in responsiveness to carnitine supplementation be observed.

4.3.5. CARNITINE PROTECTION REQUIRES THE CYTOCHROME C HEME LYASE, Cyc3p

Deletion of the cytochrome c heme lyase *CYC3*, leads to increased sensitivity compared to the wild type control on plates containing H_2O_2 (Figure 4.4 A). In addition, when compared to all other strains, the deletion of *CYC3* results in a notable decrease in the strains responsiveness to carnitine treatment. Cyc3p, a conserved eukaryotic protein, is required for the maturation of the two yeast cytochrome c's, Cyc1p and Cyc7p, through the attachment of prosthetic heme groups (Tong and Margoliash, 1998; Dumont *et al*, 1991; Schwartz and Cox, 2002). Deletion of Cyc3p results in a strain lacking mature cytochrome c. Interestingly, *CYC3* expression is induced by carnitine and not H_2O_2, whereas the expression of *CYC1* and to a lesser degree *CYC7* is induced by the presence by H_2O_2 (Table 4.4).

Table 4.4. Expression fold changes of CYC genes. Shaded blocks are used to highlight upregulation. The respective p-values are indicated in brackets

Gene Name	Expression fold change	
	Wt vs. wt +H_2O_2	H2O2 vs. H2O2 + carnitine
CYC3	-1.06 (p = 0.42)	1.99 (p = 0.084)
CYC1	2.92 (p = 0.0012)	-1.08 (p = 0.016)
CYC7	1.25 (p = 0.26)	-1.11 (p = 0.033)
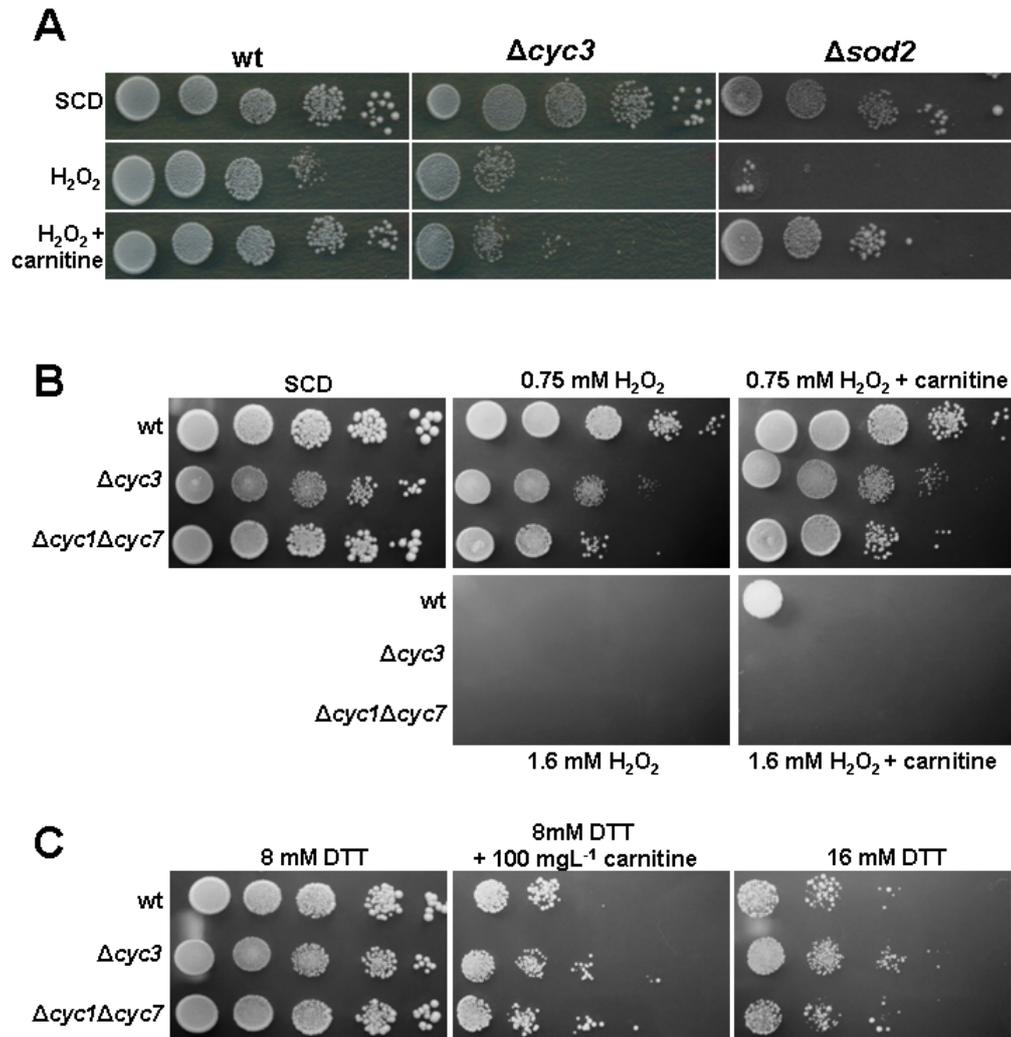
Figure 4.4. (**A**) deletion of the cytochrome c heme lyase, *CYC3*, renders the yeast strain more sensitive to H_2O_2 treatment compared to wild type and less responsive to growth enhancement by carnitine addition as compared to deletion of *SOD2* and also all other gene deletions screened. (**B**) the effect of *CYC3* deletion is comparable to a strain with no cytochrome c (Δ*cyc1*Δ*cyc7*) for carnitine's protection in the presence of H_2O_2. (**C**) *CYC3* and both cytochrome c encoding genes are enhancing carnitine's toxicity in combination with DTT.

To assess if the observed phenotype is due to the lack of functional cytochrome c, or a separate function of Cyc3p, a Δ*cyc3* strain was compared to a strain with both cytochrome c encoding genes, *CYC1* and *CYC7*, deleted. The strains were spotted on plates containing H_2O_2 and also DTT, with and without carnitine supplementation. The double mutant's sensitivity to the stress inducing agents and responsiveness to carnitine supplementation was in all cases comparable to that of the Δ*cyc3* strain (Figure 4.4 B). Both strains exhibit increased sensitivity towards H_2O_2 in comparison with the wild type strain and a decreased responsiveness to carnitine. Furthermore, both the Δ*cyc3* and the Δ*cyc1*Δ*cyc7* double mutant grow slightly better than the wild type when exposed to 16 mM DTT (Figure 4.4 C). This difference is enhanced on plates

with DTT and carnitine. The absence of functional cytochrome c therefore suppresses both carnitine-related stress phenotypes, the improvement in the presence of H_2O_2 and the detrimental impact in the presence of thiol-modifying agents. Expressing *CYC3* from the multi-copy plasmid, YEpLac195, resulted in complementation of the Δ*cyc3* strain with regards to sensitivity to oxidants and carnitine responsiveness. However, no effect was observed when the same construct was expressed in the wild type strain or the Δ*sod2*, Δ*trx2* or Δ*yap1* strains (data not shown).

4.4. DISCUSSION

A beneficial role for carnitine has been indicated in various human disease states associated with oxidative damage and mitochondrial decay (Calabrese *et al*, 2009). The molecular mechanisms behind these observations, however, remain poorly defined. In addition, a role for carnitine in protection against oxidative stress in yeast has recently been indicated (Franken *et al*, 2009). This study presents an analysis of the effect of L-carnitine supplementation in oxidative stress conditions and aims to establish the yeast, *Saccharomyces cerevisiae*, as a model system for further studies in this field.

Carnitine specifically protected yeast cells from the damaging effects of the ROS generating oxidants H_2O_2 and menadione. Interestingly, the effects of both oxidants on global gene expression were shown to be largely identical (Gasch *et al*, 2000). No or little effect was observed in the case of CHP and only a marginal difference in the case of LoaOOH induced stress, which suggests a specific and not general function for carnitine in oxidative stress protection.

Intriguingly, carnitine supplementation in combination with the thiol oxidant diamide or the thiol reducing agent DTT resulted in an enhanced toxicity. It is noteworthy that in a yeast deletion library screen of the compounds used in this study it was reported that deletion of genes involved in mitochondrial function resulted in sensitivity towards H_2O_2 and the same mutants caused resistance to diamide. As far as we are aware, this is the first report suggesting a detrimental or toxic effect of carnitine. This finding may be of significant importance when considering that DTT as well as carnitine have been proposed as therapeutic agents for the treatment of Alzheimer's and other neurodegenerative diseases (Thal *et al*, 1996; Marcum *et al*, 2005; Offen *et al*, 1996). Co-administration of these two compounds could have unexpected effects.

For both the protective and detrimental effects observed in carnitine supplemented conditions a linear correlation to carnitine concentration was observed. In all cases a

carnitine related effect was observed from concentrations of 100 mgL^{-1} (0.5 mM) upwards, in solid media. In liquid media, cells that were ptregrown at concentrations of 1 mg/l of carnitine displayed significantly better survival than cells grown in the absence of carnitine, indicating that carnitine excerts protective affects at concentrations found in natural systems. The requirement for higher carnitine levels on solid media can possibly be accounted for by limited carnitine uptake) by the carnitine carrier Agp2 which is subject to glucose repression. Carnitine uptake in these conditions was reported to be only ~ 5% of carnitine uptake under non-repressed conditions (Van Roermund *et al*, 1999; Stella *et al*, 2005). In the same studies it was also shown that carnitine added at higher concentrations (0.5 mM) could compensate for deletion of Agp2p, which may account for the concentration dependence of the carnitine phenotypes on solid glucose media. It seems therefore likely that carnitine is required at higher concentrations in solid media to provide a sufficient localized carnitine concentration around the growing colonies.

A protective effect for carnitine against the generation of intracellular ROS has been extensively described in mammalian systems (Binienda *et al*, 2006; Savitha *et al*, 2005; Sachan *et al*, 2005). Using the ROS sensitive probe DCFH-DA we show that carnitine indeed protects against ROS formation in *S. cerevisiae*. Such findings have in some cases been explained by a possible antioxidant activity of carnitine (Calo *et al*, 2006), while other studies have indicated no such activity (Rhemrev *et al*, 2000). Our data confirm that carnitine has no free radical scavenging activity when used in the ABTS decolouration assay. The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay that was used in a previous study to indicate carnitine's free radical scavenging capacity has been indicated to be a less chemically sound and valid measurement than the ABTS scavenging assay used in this study (Gulcin 2006; Huang *et al*, 2005). When using carnitine in yeast based system to screen for antioxidant potential, the molecule exhibited phenotypes strikingly similar to the phenotypes previously reported for known antioxidants. In summary, carnitine performs similar to an antioxidant in a biological context, but is not an antioxidant itself. This suggests the effect of carnitine to be mediated by genetic factors, similar to the antioxidant selenium (Rayman *et al*, 2000).

The data show that carnitine requires the central regulators *YAP1* or *SKN7* as well as genes central to metabolism of the cellular antioxidant glutathione, namely *GSH1* and *GLR1* to protect against oxidative stress. This reflects the central role of these factors in oxidative stress protections and further strengthens the assumption that the effect of

carnitine is not due to a chemical activity of the compound itself. The presence of carnitine was able to enhance growth of strains that did not produce the enzymes Sod2p, Tsa1p, and Trx2p, which function in an antioxidant capacity downstream of regulators such as Yap1p.

The effect of carnitine supplementation on global gene expression of peroxide stressed cultures was evaluated and several genes were found to be differentially expressed, confirming carnitine's involvement in the regulation of gene expression. To establish if any of the genes upregulated above a 1.8 fold cut-off is required for carnitine-dependent protection, we evaluated representative deletion strains of the identified genes on H_2O_2 and DTT containing plates for responsiveness to carnitine. Of all strains tested, only deletion of the cytochrome c heme lyase, *CYC3*, led to a notable reduction in response to carnitine. This was found to be the case for both H_2O_2 and DTT. In addition, the effect of *CYC3* deletion is similar to deletion of both cytochrome c's, *CYC1* and *CYC7*, indicating the effect to be caused by the absence of cytochrome c in the Δ*cyc3* strain. Since deletion of *CYC3* leads to a reduced responsiveness to carnitine and multi-copy expression has no discernable effect it can be concluded that *CYC3* is required for growth enhancement by carnitine, but not exclusively involved in the effect of carnitine. Interestingly, both *CYC1* and *CYC7* are upregulated by H_2O_2 stress, but *CYC3* expression is only induced once carnitine is added to the media. If the function of *CYC3* is rate limiting under these conditions, its increased expression would explain its contribution to the protection of carnitine. In general, the integrity of the electron transport chain is required for resistance to H_2O_2. Increased cytochrome c content by enhancing expression of *CYC3* or *CYC1* and *CYC7* can specifically be of benefit during oxidative stress conditions by (i) playing a role in the regulation of apoptosis caused by oxidative damage (Eisenberg *et al*, 2007), (ii) a suggested antioxidant function of cytochrome c itself (Forman and Azzi, 1997; Skulachev, 1998) and (iii) its role in the respiratory chain helping to keep H_2O_2 and superoxide at a lower physiological level (Zhao *et al*, 2003).

Previous data have established a positive effect of carnitine on mitochondrial metabolism. This has been suggested to be caused by the stimulatory effect of the carnitine shuttle on mitochondrial functions (Hagen *et al*, 1998; Zhu *et al*, 2008). The results of this study present the first report that carnitine may act more specifically through other genetic systems, specifically by inducing the expression of *CYC3*, which directly impacts on mitochondrial function and also on resistance to oxidative stresses.

Future work will focus on the further elucidation of the role played by *CYC3* in this context and also the identification of upstream factors involved in the regulation of the effects carnitine has on cellular physiology.

4.5. REFERENCES

Abdul, H. M., Calabrese, V., Calvani, M. and Butterfield, D. A. (2006). Acetyl-L-carnitine-induced up-regulation of heat shock proteins protects cortical neurons against amyloid-beta peptide 1-42-mediated oxidative stress and neurotoxicity: implications for Alzheimer's disease. *J Neurosci Res* **84,** 398-408.

Berben, G.J., Dumont, J., Gilliquet, V., Bolle P.A. and Hilger, F. (1991). The Ydp plasmids: a uniform set of vectors bearing versatile gene disruption cassettes for *Saccharomyces cerevisiae*. *Yeast* **7,** 475-477.

Bieber, L. L. (1988). Carnitine. *Annu Rev Biochem* **57,** 261-83.

Binienda, Z. K., Ali, S. F., Virmani, A., Amato, A., Salem, N. and Przybyla, B. D. (2006). Co-regulation of dopamine D1 receptor and uncoupling protein-2 expression in 3-nitropropionic acid-induced neurotoxicity: neuroprotective role of L-carnitine. *Neurosci Lett* **410,** 62-5.

Boer, V. M., de Winde, J. H., Pronk, J. T. and Piper, M. D. (2003). The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. *J Biol Chem* **278,** 3265-74.

Calabrese, V., Giuffrida Stella, A. M., Calvani, M. and Butterfield, D. A. (2006). Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes. *J Nutr Biochem* **17,** 73-88.

Calabrese, V., Ravagna, A., Colombrita, C., Scapagnini, G., Guagliano, E., Calvani, M., Butterfield, D. A. and Giuffrida Stella, A. M. (2005). Acetylcarnitine induces heme oxygenase in rat astrocytes and protects against oxidative stress: involvement of the transcription factor Nrf2. *J Neurosci Res* **79,** 509-21.

Calabrese, V., Ravagna, A., Colombrita, C., Scapagnini, G., Guagliano, E., Calvani, M., Butterfield, D. A. and Giuffrida Stella, A. M. (2005). Acetylcarnitine induces heme oxygenase in rat astrocytes and protects against oxidative stress: involvement of the transcription factor Nrf2. *J Neurosci Res* **79,** 509-21.

Calo, L. A., Pagnin, E., Davis, P. A., Semplicini, A., Nicolai, R., Calvani, M. and Pessina, A. C. (2006). Antioxidant effect of L-carnitine and its short chain esters: relevance for the protection from oxidative stress related cardiovascular damage. *Int J Cardiol* **107,** 54-60.

Chae, H. Z., Chung, S. J. and Rhee, S. G. (1994). Thioredoxin-dependent peroxide reductase from yeast. *J Biol Chem* **269,** 27670-8.

De Beer, D., Joubert, E., Gelderblom, W. C. and Manley, M. (2003). Antioxidant activity of South African red and white cultivar wines: free radical scavenging. *J Agric Food Chem* **51,** 902-9.

Delaunay, A., Pflieger, D., Barrault, M. B., Vinh, J. and Toledano, M. B. (2002). A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell* **111,** 471-81.

Dumont, M. E., Cardillo, T. S., Hayes, M. K. and Sherman, F. (1991). Role of cytochrome c heme lyase in

mitochondrial import and accumulation of cytochrome c in *Saccharomyces cerevisiae*. *Mol Cell Biol* **11,** 5487-96.

Edgar, R., Domrachev, M. and Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* **30,** 207-10.

Eisenberg, T., Buttner, S., Kroemer, G. and Madeo, F. (2007). The mitochondrial pathway in yeast apoptosis. *Apoptosis* **12,** 1011-23.

Evans, M. V., Turton, H. E., Grant, C. M. and Dawes, I. W. (1998). Toxicity of linoleic acid hydroperoxide to Saccharomyces cerevisiae: involvement of a respiration-related process for maximal sensitivity and adaptive response. *J Bacteriol* **180,** 483-90.

Forman, H. J. and Azzi, A. (1997). On the virtual existence of superoxide anions in mitochondria: thoughts regarding its role in pathophysiology. *Faseb J* **11,** 374-5.

Franken, J., Kroppenstedt, S., Swiegers, J. H. and Bauer, F. F. (2008). Carnitine and carnitine acetyltransferases in the yeast *Saccharomyces cerevisiae*: a role for carnitine in stress protection. *Curr Genet* **53,** 347-60.

Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D. and Brown, P. O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* **11,** 4241-57.

Grant, C. M. (2001). Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. *Mol Microbiol* **39,** 533-41.

Gulcin, I. (2006). Antioxidant and antiradical activities of L-carnitine. *Life Sci* **78,** 803-11.

Hagen, T. M., Ingersoll, R. T., Wehr, C. M., Lykkesfeldt, J., Vinarsky, V., Bartholomew, J. C., Song, M. H. and Ames, B. N. (1998). Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. *Proc Natl Acad Sci U S A* **95,** 9562-6.

Huang, D., Ou, B. and Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *J Agric Food Chem* **53,** 1841-56.

Jakubowski, W. and Bartosz, G. (1997). Estimation of oxidative stress in *Saccharomyces cerevisae* with fluorescent probes. *Int J Biochem Cell Biol* **29,** 1297-301.

Jamieson, D. J. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast* **14,** 1511-27.

Kispal, G., Sumegi, B., Dietmeier, K., Bock, I., Gajdos, G., Tomcsanyi, T. and Sandor, A. (1993). Cloning and sequencing of a cDNA encoding *Saccharomyces cerevisiae* carnitine acetyltransferase. Use of the cDNA in gene disruption studies. *J Biol Chem* **268,** 1824-9.

Kunau, W. H., Dommes, V. and Schulz, H. (1995). beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. *Prog Lipid Res* **34,** 267-342.

Maeta, K., Nomura, W., Takatsume, Y., Izawa, S. and Inoue, Y. (2007). Green tea polyphenols function as prooxidants to activate oxidative-stress-responsive transcription factors in yeasts. *Appl Environ Microbiol* **73,** 572-80.

Marcum, J. L., Mathenia, J. K., Chan, R. and Guttmann, R. P. (2005). Oxidation of thiol-proteases in the hippocampus of Alzheimer's disease. *Biochem Biophys Res Commun* **334,** 342-8.

Offen, D., Ziv, I., Sternin, H., Melamed, E. and Hochman, A. (1996). Prevention of dopamine-induced cell death by thiol antioxidants: possible implications for treatment of Parkinson's disease. *Exp Neurol* **141,** 32-9.

Ohtake, Y. and Yabuuchi, S. (1991). Molecular cloning of the gamma-glutamylcysteine synthetase gene of *Saccharomyces cerevisiae*. *Yeast* **7,** 953-61.

Palmieri, L., Lasorsa, F. M., De Palma, A., Palmieri, F., Runswick, M. J. and Walker, J. E. (1997). Identification of the yeast *ACR1* gene product as a succinate-fumarate transporter essential for growth on ethanol or acetate. *FEBS Lett* **417,** 114-8.

Pedrajas, J. R., Kosmidou, E., Miranda-Vizuete, A., Gustafsson, J. A., Wright, A. P. and Spyrou, G. (1999). Identification and functional characterization of a novel mitochondrial thioredoxin system in *Saccharomyces cerevisiae*. *J Biol Chem* **274,** 6366-73.

Rayman, M. P. (2000). The importance of selenium to human health. *Lancet* **356,** 233-41.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* **26,** 1231-7.

Reddy, J. K. and Mannaerts, G. P. (1994). Peroxisomal lipid metabolism. *Annu Rev Nutr* **14,** 343-70.

Rhemrev, J. P., van Overveld, F. W., Haenen, G. R., Teerlink, T., Bast, A. and Vermeiden, J. P. (2000). Quantification of the nonenzymatic fast and slow TRAP in a postaddition assay in human seminal plasma and the antioxidant contributions of various seminal compounds. *J Androl* **21,** 913-20.

Sachan, D. S., Hongu, N. and Johnsen, M. (2005). Decreasing oxidative stress with choline and carnitine in women. *J Am Coll Nutr* **24,** 172-6.

Saffi, J., Sonego, L., Varela, Q. D. and Salvador, M. (2006). Antioxidant activity of L-ascorbic acid in wild-type and superoxide dismutase deficient strains of *Saccharomyces cerevisiae*. *Redox Rep* **11,** 179-84.

Savitha, S., Tamilselvan, J., Anusuyadevi, M. and Panneerselvam, C. (2005). Oxidative stress on mitochondrial antioxidant defense system in the aging process: role of DL-alpha-lipoic acid and L-carnitine. *Clin Chim Acta* **355,** 173-80.

Schmalix, W. and Bandlow, W. (1993). The ethanol-inducible *YAT1* gene from yeast encodes a presumptive mitochondrial outer carnitine acetyltransferase. *J Biol Chem* **268,** 27428-39.

Schmitt, M. E., Brown, T. A. and Trumpower, B. L. (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res* **18,** 3091-2.

Schwarz, Q. P. and Cox, T. C. (2002). Complementation of a yeast CYC3 deficiency identifies an X-linked mammalian activator of apocytochrome c. *Genomics* **79,** 51-7.

Silva-Adaya, D., Perez-De La Cruz, V., Herrera-Mundo, M. N., Mendoza-Macedo, K., Villeda-Hernandez, J., Binienda, Z., Ali, S. F. and Santamaria, A. (2008). Excitotoxic damage, disrupted energy metabolism, and oxidative stress in the rat brain: antioxidant and neuroprotective effects of L-carnitine. *J Neurochem* **105,** 677-89.

Skulachev, V. P. (1998). Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Lett* **423,** 275-80.

Stella, C.A., Burgos, H.I., Salellas, M.L., Cristaldo, M.L., Ramos, E.H., Kriguer, N. (2005). L-Carnitine effect upon iron growth inhibition on *Saccharomyces cerevisiae*. *Letters in Drug Design and Discovery* **2,** 44-47.

Swiegers, J. H., Dippenaar, N., Pretorius, I. S. and Bauer, F. F. (2001). Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: three carnitine acetyltransferases are essential in a carnitine-dependent strain. *Yeast* **18,** 585-95.

Swiegers, J. H., Vaz, F. M., Pretorius, I. S., Wanders, R. J. and Bauer, F. F. (2002). Carnitine biosynthesis in *Neurospora crassa*: identification of a cDNA coding for epsilon-N-trimethyllysine hydroxylase and its functional expression in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **210,** 19-23.

Thal, L. J., Carta, A., Clarke, W. R., Ferris, S. H., Friedland, R. P., Petersen, R. C., Pettegrew, J. W., Pfeiffer, E., Raskind, M. A., Sano, M., Tuszynski, M. H. and Woolson, R. F. (1996). A 1-year multicenter placebo-controlled study of acetyl-L-carnitine in patients with Alzheimer's disease. *Neurology* **47,** 705-11.

Thorpe, G. W., Fong, C. S., Alic, N., Higgins, V. J. and Dawes, I. W. (2004). Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes. *Proc Natl Acad Sci U S A* **101,** 6564-9.

Tong, J. and Margoliash, E. (1998). Cytochrome c heme lyase activity of yeast mitochondria. *J Biol Chem* **273,** 25695-702.

van Roermund, C. W., Elgersma, Y., Singh, N., Wanders, R. J. and Tabak, H. F. (1995). The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. *Embo J* **14,** 3480-6.

Van Roermund, C. W., Hettema, E. H., Van Den Berg, M., Tabak, H. F. and Wanders, R. J. (1999). Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine transporter, Agp2p. *EMBO J* **18,** 5843-52.

Veal, E. A., Ross, S. J., Malakasi, P., Peacock, E. and Morgan, B. A. (2003). Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor. *J Biol Chem* **278,** 30896-904.

Zhao, Y., Wang, Z. B. and Xu, J. X. (2003). Effect of cytochrome c on the generation and elimination of O^{2*-} and H_2O_2 in mitochondria. *J Biol Chem* **278,** 2356-60.

Zhu, X., Sato, E. F., Wang, Y., Nakamura, H., Yodoi, J. and Inoue, M. (2008). Acetyl-L-carnitine suppresses apoptosis of thioredoxin 2-deficient DT40 cells. *Arch Biochem Biophys* **478,** 154-60.

Zyracka, E., Zadrag, R., Koziol, S., Krzepilko, A., Bartosz, G. and Bilinski, T. (2005). Ascorbate abolishes auxotrophy caused by the lack of superoxide dismutase in *Saccharomyces cerevisiae*. Yeast can be a biosensor for antioxidants. *J Biotechnol* **115,** 271-8.

Chapter 5

RESEARCH RESULTS III

Effect of carnitine supplementation on genome wide expression in the yeast *Saccharomyces cerevisiae*.

5.1. INTRODUCTION

In eukaryotes the primary function of L-carnitine is as a shuttling molecule, transferring the intermediates of energy metabolism between intracellular compartments (Bremer, 1983). Carnitine has been shown to have therapeutic potential, due to its stimulatory action on mitochondrial metabolism and also by balancing limited and compartmentalized pools of CoA and Acyl-CoA. Aside from carnitine's central role in energy metabolism, several studies have reported beneficial effects associated with carnitine supplementation at concentrations above physiological levels that appear to be regulated by genetic factors. For instance, carnitine's beneficial effect in neurodegenerative conditions, such as Alzheimer's disease, have recently been proposed to be mediated by the transcriptional induction of factors such as heat shock proteins and HO-1 that are associated with cellular stress protection (Calabrese *et al*, 2006). Furthermore, carnitine has been shown to have regulatory consequences on the induction of apoptosis by directly interacting with the Fas receptor system, inhibition of caspases 3, 7 and 8 and also inhibiting the mitochondrial permeability transition (Moretti *et al*, 1998; Mutomba *et al*, 2000; Pastorino *et al*, 1993). The mechanism by which these effects are achieved remains to be defined.

Recently, carnitine has been shown to have a protective effect on yeast cultures during oxidative and organic acid stresses (Franken *et al*, 2008). The effect of carnitine supplementation was further investigated in chapter 4 of this thesis and shown to require the presence of the major factors, Yap1p and Skn7p, involved in oxidative stress protection. Furthermore, in an initial microarray based screen of genes upregulated by carnitine supplementation in cultures exposed to H_2O_2, a role for the cytochrome c heme lyase, Cyc3p, and the two yeast cytochrome c's, Cyc1p and Cyc7p, was described in the mediation of carnitine related effects during redox related stresses. Although the involvement of cytochrome c could indicate mediation of apoptotic processes, this hypothesis still needs to be substantiated.

This work presents a detailed analysis of the effect of carnitine supplementation on genome wide transcription. Microarray analysis was performed on growing cultures of wild type (BY4742) cells, with and without carnitine supplementation, and also similar cultures exposed to oxidative stress induced by the addition of H_2O_2. The results of the data generated by DNA microarrays were analyzed by assessing enrichment of

functional classification within sets of differentially expressed genes and also by cluster analysis of the data derived from the microarray analysis. In addition, possible links to apoptotic pathways were investigated by projecting the expression data onto known interaction pathways. The result of this analysis lays the foundation for further studies on carnitine's role in redox related stresses.

5.2. MATERIALS AND METHODS

5.2.1. MICROARRAY ANALYSIS

Cultures of the wild type strain BY4742 were grown overnight in SCD containing tubes to serve as pre-cultures. Two flasks containing 50 ml of freshly prepared SCD media and also two containing SCD with carnitine (1000 mgL^{-1}) was inoculated to an O.D.$_{600}$ ~ 0.1 and grown to mid-log phase (O.D.$_{600}$ 0.4 -.0.5). A duplicate set was also inoculated and exposed to 0.4 mM H_2O_2 for 30 min before harvesting and isolation of total RNA. RNA was isolated as previously described (Schmitt *et al*, 1990). The quality of total RNA, cDNA, cRNA and fragmented cRNA were confirmed using the Agilent Bioanalyzer 2100. Probe preparation and hybridization to Affymetrix Genechip® microarrays were performed according to Affymetrix instructions, starting with 6 µg of total RNA. Results for each condition were derived from 2 independent culture replicates. Gene chips were scanned using GeneChip® Scanner 3000. Acquisition of array images and were performed using Affymetrix GeneChip® Operating Software (GCOS) version 1.4. Before determination of induction or repression of each gene spot intensities were normalized using GC-RMA (Wu and Irazarry, 2004). The fold induction or repression was calculated as the ratio between carnitine treated and non-treated cultures for both stresses and non-stressed conditions. Genes that changed at least 1.5 fold were considered for further analysis. The data discussed in this study have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE16346 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE16346). Statistical analysis of functional groups was performed by using FUNSPEC (Robinson *et al*, 2002). Cluster analysis was performed using Cluster v 2.12 and visualized using Treeview v 1.6 (Eisen *et al*, 1998). Clusters were analyzed for enrichment of functional groups using FUNSPEC (Robinson *et al*, 2002). Centroid linkage hierarchical clustering was performed according to recommendations of the

Cluster v 2.12 software manual, except for using log-untransformed data (since the array data was not generated using a two-dye system).

5.3. RESULTS AND DISCUSSION

5.3.1. EFFECT OF NORMALIZATION METHOD CHOICE

In the initial analysis of the microarray data described in chapter 4, the expression values was calculated using the Affymetrix GeneChip® Operating Software (GCOS) version 1.4, which calculates expression based on a weighed median (Affymetrix: Microarray Suite User Guide http://www.affymetrix.com/support/technical/manuals.affx). The outcome of this analysis presented difficulties in the interpretation of data for signals of a lower intensity, which constitute the majority of genes affected by carnitine supplementation. Although the data could be used to generate the screenable set of genes described in chapter 4, the variances in expression values, especially below 2 fold differential expressions, prevents the generation of a large enough set of genes which can be used for analysis such as clustering or enrichment of functional categories. Therefore, the generated arrays were re-normalized using GC-RMA for further analysis (Wu and Irazarry, 2004). This method is founded on a model-based algorithm, which incorporates the expression of multiple microarrays to calculate the expression of a specific gene, compared to the use of one microarray for normalization in GCOS. The use of such a model based algorithm can be expected to generate more reproducible results, since probe response patterns are fitted over multiple arrays, which detects and excludes abnormally behaving probes. The outcome of this normalization method generally provided less variance in expression values and results in the generation of larger usable gene sets that are significantly induced or repressed. In addition, the use of GC-RMA for normalization enabled the use of a lower fold expression cut-off value at 1.5.

5.3.2. DIFFERENTIAL GENE EXPRESSION EFFECTED BY CARNITINE SUPPLEMENTATION IN YEAST

To establish the effect of carnitine supplementation on genome wide transcription, microarray analysis was performed on cultures of the wild type strain BY4742 that were grown in minimal media to mid-log phase and also a separate culture set that was

grown in similar media supplemented with carnitine (1000 mgL^{-1}). In addition, a set of similarly treated yeast cultures (with and without carnitine supplementation) was exposed to 4 mM H_2O_2 to induce oxidative stress. The data from this experiment was used to asses the effect of carnitine on global gene transcription in conditions of oxidative stress. Enrichments of functional categories for genes which are up and downregulated, when comparing wild-type cultures to carnitine supplemented cultures are presented in Table 5.1. Table 5.2 lists enriched categories when H_2O_2 treated cultures with and without carnitine are compared. To distinguish between the effect of carnitine and the effect of H_2O_2 addition, the genes that were found to be differentially expressed by the presence of carnitine in conditions of oxidative stress were aligned and clustered in comparison with the expression values for the same genes under normal growing conditions (wt) and carnitine supplemented conditions (wt + C) (Figure 5.1 and Table 5.3).

Carnitine supplementation resulted in the induction of 41 genes and the repression of 86 transcripts ($\geq$ 1.5 fold). Genes included in the functional categories of dipeptide transport (GO:0042938; p-value, 0.0001124) and regulation of DNA metabolism (GO:0051052; p-value, 3.543e-05) were represented among the genes induced by carnitine. Also included in the set of upregulated genes are cell wall associated genes (GO:0009277; p-value, 7.001e-06), which contains a group of cell wall mannoproteins of the Srp1p/Tip1p family (*DAN1*, *TIR2*, *TIR3*, *TIR4*) that have been shown to be induced by cellular stresses (Kowalski *et al*, 1995; Cohen *et al*, 2001). The genes that were downregulated by carnitine supplementation group into two main categories, one of which is a group associated with metal homeostasis including the functional groupings of iron ion binding (GO:0005506; p-value, 0.0001011), iron ion transport (GO:0006811; p-value, 1.853e-05) and copper ion transport ([GO:0015677; p-value, 1.805e-06). A second group of functionally enriched genes are associated with the metabolism of reserve energy carbohydrates and complex sugars and include the functional classifications of metabolism of energy reserves (e.g. glycogen and trehalose) (MIPS: 02.19; p-value, 6.874e-06), sugar, glycoside, polyol and carboxylate metabolism (MIPS: 01.05.02.07; p-value, 0.0006086) and maltose metabolism (GO:0000023; p-value, 0.0004312). Furthermore, the downregulated gene set also contains the functional categories of phosphate transport (GO:0005315; p-value, 0.0002397) and membrane fusion (GO:0006944, p-value, 0.0008668).

Table 5.1. Functions overrepresented for wild type *vs* wild type + carnitine.

Classification	p	Genes	Source[a]
Upregulated			
Catabolism of nitrogenous compounds	0.0003718	*DAL7 DAL3*	1
Regulation of DNA metabolic process	3.543e-05	*NSE4 RAD55 PSY3*	3
Dipeptide transport	0.0001124	*DAL5 PTR2*	3
Fungal-type cell wall	7.001e-06	*FIT1 TIR3 DAN1 YGP1 HPF1 TIR4 TIR2*	3
Downregulated			
Metabolism of energy reserves (e.g. glycogen, trehalose)	6.874e-06	*MAL32 GLC3 GIP2 GSY1 YGR287C YIL172C PGM2 ATH1*	1
Sugar, glucoside, polyol and carboxylate catabolism	0.0006086	*GAL7 MAL32 XKS1 YGR287C YIL172C PGM2 ATH1*	1
Heavy metal ion transport (Cu+, Fe3+, etc.)	0.0001728	*FRE2 FRE1 PHO84 FET4 CTR1*	1
Phosphate transport	0.0003264	*PHO89 PIC2 PHO84*	2
Iron ion binding	0.0001011	*GAL7 CYC7 AIM17 ARN2 FRE2 FRE1 FET4 ENB1 FRE3 FIT2 PDH1*	3
Iron ion transport	4.111e-06	*ARN2 FRE2 FRE1 FET4 ENB1 FRE3 FIT2*	3
Copper ion import	1.805e-06	*FRE2 FRE1 FET4 CTR1*	3
Carbohydrate metabolic process	9.222e-06	*GAL7 MAL32 GLC3 XKS1 YGR287C YIL172C PGM2 YNR034W-A MDH2 YPL014W ATH1*	3
Ion transport	1.853e-05	*HSP30 ARN2 SPL2 FRE2 FRE1 FET4 ENB1 FRE3 FIT2 CTR1*	3
Maltose metabolic process	0.0004312	*MAL32 YGR287C MAL11 YIL172C*	3
Membrane fusion	0.0008668	*SPL2 PHO84 CTR1*	3

[a]Source refers to classification source: 1, MIPS functional classification; 2, GO molecular function; 3, GO biological process. Classifications with p< 0.005 are shown.

Genes in cluster A (Figure 5.2; Table 5.3) were also enriched in the functional category of iron homeostasis (GO:0006879; p-value, 0.001509). Genes in this cluster were generally induced by H_2O_2, whereas carnitine addition lowered expression in both wt and also H_2O_2 treated cultures. Iron homeostasis plays a central role in oxidative and specifically H_2O_2 induced stresses, since transition metals such as Fe^{2+} catalyze the formation of highly reactive hydroxyl radicals via the Fenton reaction (Halliwel and Aruoma, 1991; Perrone *et al*, 2008). Included in this group of genes is the transcription factor *AFT2* (which regulates genes involved in iron homeostasis and oxidative stress), *ISU1* (which performs a scaffolding function during assembly of iron-sulfur clusters and interacts physically and functionally with yeast frataxin, Yfh1p), *MMT1* (involved in

mitochondrial iron accumulation), *NFU1* (required for the maturation of Fe/S clusters). Interestingly, carnitine has previously been shown to protect yeast cells against exposure to high iron concentrations (Stella *et al*, 2005). It would certainly be of interest to further investigate the impact of carnitine on cellular iron homeostasis.

Additional groupings in this cluster (A), which share similar expression patterns, includes the cellular responses to toxins (GO:0009636; p-value, 7.132e-08) and reactive oxygen species (GO:0000302; p-value, 0.0002457) and also genes involved in autophagy (GO:0006914; p-value, 0.000387). Furthermore, two genes from the y-AP1 family, *YAP7* and *YAP4/CIN5*, which regulate transcription in response to environmental stresses, are included in cluster A.

Under conditions of oxidative stress carnitine supplementation increased the expression of 290 gene products and resulted in decreased expression of 303 genes ($\geq$ 1.5 fold). An interesting scenario presents itself in the categories of genes induced by carnitine under oxidative stress, in the sense that a prominent feature of this group involves enrichment of functional categories involved in carbon and carbohydrate and reserve energy metabolism [regulation of c-compound and carbohydrate metabolism (MIPS: 01.05.25, p-value, 0.0001137), carbohydrate transport (GO:0008643; p-value, 6.002e-06), carbohydrate metabolic process (GO:0005975; p-value, 2.419e-05) and glycogen catabolism (GO:0005980; p-value, 8.388e-05)]. This is interestingly reminiscent of the categories regulated by carnitine in the absence of an oxidative stress signal, except that genes involved in glycogen synthesis and accumulation were downregulated in wt cultures, whereas glycogen catabolic genes were upregulated during oxidative stress by the presence of carnitine. A group of genes involved in glycogen metabolism is also represented in cluster B, which contains genes that are upregulated by carnitine, mostly in an oxidative stress dependent manner. Included in this set of genes are *GPH1* (required for the mobilization of glycogen and expression regulated by stress-response elements and by the HOG MAP kinase pathway), *GIP2* and *GAC1*. The latter two genes are also included in the categories enriched from genes induced by carnitine under oxidative stress and form part of the protein phophatase I (PPI) complex (GO:0000164; p-value, 0.0001149), which includes *REG2*, encoding a protein involved in glucose repression, *GIP2* and *PIG1*, which are both involved the regulation of glycogen metabolism and also *GAC1*, which is the PPI regulatory subunit that mediates glycogen metabolism and also the induction of genes involved in the response to heat shock.

Table 5.2. Functions overrepresented for wild type treated with H_2O_2 without carnitine vs. with carnitine.

Classification	p	Genes	Source[a]
Upregulated			
Regulation of C-compound and carbohydrate metabolism	0.0001137	*REG2 MAL33 ADY2 SNF3 GAL3 MTH1 GIP2 MIG1 MIG2 RPI1 PIG1 CAT8 ATO2 RAS1 GAC1 SWI1*	1
Carbohydrate transport	6.002e-06	*MAL31 SNF3 HXT7 MIG2 MAL11 HXT4 HXT5 YKR075C HXT2 HXT11*	2
Carbohydrate metabolic process	2.419e-05	*MAL33 GAL3 MIG1 AMS1 MIG2 GUT1 HXT4 YHR210C SGA1 RPI1 GUT2 XYL2 PGM2 CAT8 YNR071C HPF1 GPH1 GDB1*	2
Transmembrane transport	3.158e-05	*MAL31 SNF3 HXT7 MAL11 HXT4 HXT5 HXT2 HXT11*	2
Glycogen catabolic process	8.288e-05	*SGA1 GPH1 GDB1*	2
Proline catabolic process	0.0003246	*PUT3 PUT1 PUT4*	2
Protein phosphatase type 1 complex	0.0001149	*REG2 GIP2 PIG1 GAC1*	4
RNA polymerase I transcription factor complex	0.000785	*RRN7 RRN3 RRN11*	4
Downregulated			
biosynthesis of arginine	1.89e-08	*ARG5,6 ARG4 ARG3 CPA2 ECM40 ARG1 ARG8 ORT1*	1
mating	5.866e-06	*FIG1 FUS1 KAR4 HO SPR3 PRM2 PRM10 SAG1 BFA1 PRM6 KAR5 SCW10 AGA1 PRM3*	1
purine nucleotide anabolism	5.66e-05	*ADE1 HIS7 HIS4 ADE5,7 ADE6 ADE17 ADE12 ADE2*	1
metabolism of nonprotein amino acids	4.102e-05	*YAT2 ARG5,6 ARG3 ECM40 ARG8*	1
iron-sulfur cluster binding	0.0001424	*GLT1 ACO2 ECM17 NFU1 ISA1 ACO1 ISU1 PDH1*	2
catalytic activity	0.0001136	*ADE1 ACH1 GAL7 MET8 PYC2 HIS7 HIS4 YCR102C GPM2 GLT1 ALT2 SUR2 ARO10 PHM8 ARG5,6 STR3 ADE5,7 ADE6 SER2 SPO11 ARG4 DOG1 LYS1 INO1 URA8 CPA2 ECM17 MAE1 URA1* YLL056C *SHM2* YLR460C *ECM40 ADE17 GAS3 SCW10 BIO3 GPD2 ARG8 LEU9 ADE2* YPL033C	2
aromatic compound metabolic process	3.01e-11	*PYC2 GCV1 ARG5,6 MET13 LYS1 ARG3 CPA2 ECM17 DRE2 ECM40 SNO1 GCV2 NRK1 ARG1 GPD2 ADE2 ORT1 SSU1*	2
amino acid biosynthetic process	1.29e-08	*MET8 HIS7 HIS4 GLT1 HOM3 HIS1 ARG5,6 STR3 SER2 ARG4 LYS1 ARG3 CPA2 ECM17 MET1 ECM40 ARG1 ARG8 LEU9 ORT1 MET16*	2
organic acid metabolic process	3.037e-07	*PYC2 ARG5,6 YJR111C ECM17 MET1 CTF13 ARG1 ORT1*	2
sexual reproduction	3.71e-07	*FIG1 FUS1 KAR4 PCL2 GIC2 PRM6 FUS2 PRM1 AGA1*	2
response to toxin	4.33e-05	YCR102C *YDR132C ATF2 RTA1* YKL070W YLL056C YLR108C YLR346C YLR460C *YNL260C* YOL163W *RSB1 MET16*	2
propionate metabolic process	0.0001363	*ACO1 PDR12 CIT3 PDH1*	2

[a]Source refers to classification source: 1, MIPS functional classification; 2, GO molecular function; 3, GO biological process; 4, GO cellular component. Classifications with p< 0.005 are shown.
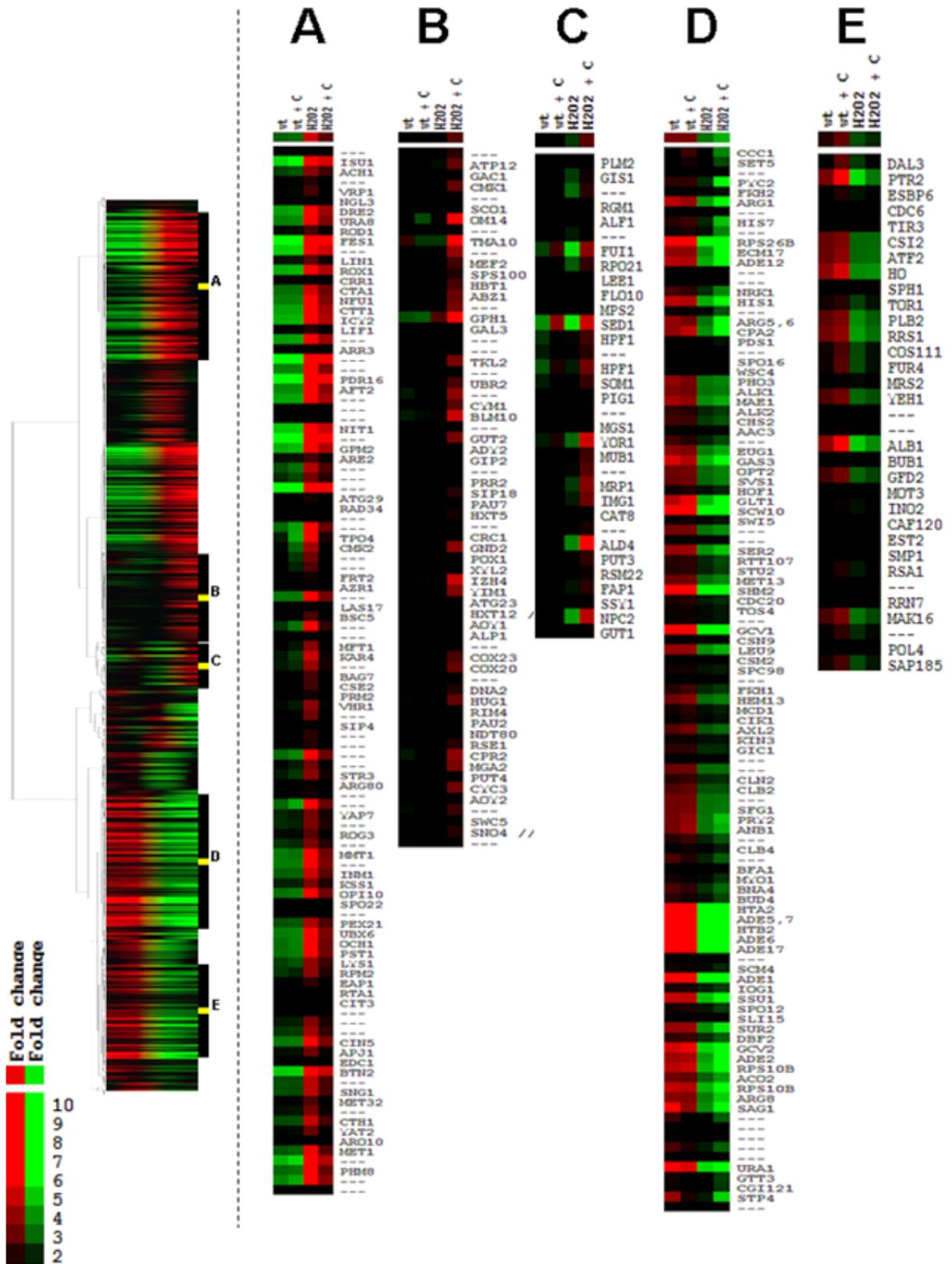
Figure 5.1. Clustered display of data from all genes differentially expressed (using a 1.5 fold cut-off) by carnitine addition under conditions of oxidative stress that is aligned with wild type cultures and wild type cultures supplemented with carnitine. Each gene is represented by a single coloured line, with red colouring indicating up and green downregulation, and each condition is represented by a single column. Five separate clusters are indicated by bars (**A-E**), the enriched classes for the genes in each cluster is presented in Table 5.3.

Table 5.3. Functions overrepresented in clusters A-E.

Cluster	Classification	P	Genes	Source[a]
A	Response to toxin	7.132e-08	YCR102C YDR132C *RTA1* YKL070W YLL056C YLR108C YLR346C YLR460C YNL260C YOL163W	2
	Response to ROS	0.0002457	*CTA1 CTT1*	3
	Autophagy	0.000387	*FES1* YDR132C *CTH1 ARO10 STR3 VEL1 PEF1* YKL070W YLR108C *APJ1* YNR068C *ATG29*	3
	Cellular iron ion homeostasis	0.001509	*CTH1 NFU1 MMT1 ISU1 AFT2*	3
B	Water transport	0.0002671	*AQY2 AQY1*	3
	Glycogen metabolic process	0.000974	*GIP2 GAC1 GPH1*	3
	Fatty acid metabolic process	0.003371	*POX1 MGA2 CRC1*	3
	Metabolic process	0.003652	*TKL2* YDL144C *GAL3* YDR018C *POX1 GND2 LAS21 XYL2 YIM1 ABZ1*	3
C	Metal ion binding	0.0002795	*RPO21 GIS1 MRP1 PUT3 MUB1 RGM1 CAT8 FAP1 MGS1 LEE1*	3
	DNA binding	0.006955	*RPO21 GIS1 PLM2 PUT3 MUB1 RGM1 CAT8 MGS1*	2
D	Aromatic compound metabolism	8.744e-10	*PYC2 GCV1 ARG5,6 MET13 CPA2 ECM17 GCV2 NRK1 ARG1 ADE2 SSU1*	2
	Amino acid biosynthetic process	6.518e-06	*HIS7 GLT1 HIS1 ARG5,6 SER2 CPA2 ECM17 ARG1 ARG8 LEU9*	3
	Glycine metabolic process	1.501e-05	*GCV1 SHM2 GCV2*	3
	Purine nucleotide biosynthesis	2.423e-05	*ADE1 ADE5,7 ADE6 ADE17 ADE12 ADE2*	3
	Cell cycle	0.0004786	*ALK2 MCD1 PDS1 ALK1 CDC20 SCM4 DBF2 BFA1 BUD4 CLB4 HOF1 IQG1 CLN2 CLB2*	3
E	Ribosome biogenesis	2.108e-05	*MAK16* YBL081W *FUR4 GFD2 ATF2 RRN7 SAP185 ALB1 TOR1* YEH1 RRS1 YPL068C *RSA1*	3
	RNA modification	7.992e-05	*FUR4 GFD2 HO MRS2* YPL068C *RSA1*	3
	rRNA metabolic process	0.0001021	YBL081W *FUR4 GFD2 ATF2 RRN7* YEH1 YPL068C *RSA1*	3
	Transcription from RNA polymerase I promoter	0.006239	*FUR4 RRN7 MOT3*	3

[a]Source refers to classification source: 1, MIPS functional classification; 2, GO molecular function; 3, GO biological process; 4, GO cellular component. Classifications with p< 0.005 are shown.

Genes involved in the utilization of proline (GO:0006562; p-value0.0003246) are also upregulated under carnitine supplemented conditions during oxidative stress. The three genes in this grouping are *PUT4*, an integral membrane proline transporter, *PUT1*, which catalyzes the initial step in proline catabolism and *PUT3*, a

transcriptionalregulator of genes involved in proline degradation. Proline is degraded by this pathway to glutamate, which can either feed into the TCA cycle as α-ketogluterate or act as a precursor for the biosynthesis of the cellular antioxidant glutathione (Takagi, 2008).

Proline, along with glycerol and glycogen, is considered to play an integral role in cellular stress protection. Interestingly the genes encoding the enzymes required for the degradation of glycerol, *GUT1* and *GUT2*, are also upregulated by carnitine during oxidative stress. These enzymes form part of the glycerol-3-phosphate dehydrogenase shuttle, which presents an alternative means, aside from the electron transport chain, for the oxidation of glycolytic NADH without the production of O^{2-} (Larsson *et al*, 1998). *GPD2*, which also forms part of this shuttle, is downregulated by the presence of carnitine during oxidative stress (Table 5.2).

The genes involved in glycerol degradation are represented in the functional category of carbohydrate metabolic process (GO:0005975; p-value, 2.419e-05), which includes the central transcriptional regulators of carbon metabolism, *MIG1*, *MIG2* and *CAT8* and also *RPI1*, a putative transcriptional regulator which, when overexpressed, suppresses the heat shock sensitivity of *RAS2* overexpression (Kim and Powers, 1991). *CAT8* is also represented in cluster C, made up of genes induced by carnitine in wild-type and H_2O_2 treated cultures. This cluster contains several genes coding for metal dependent DNA binding factors, which include *PUT3*, *RPO21* (largest subunit, B220, of RNA polymerase II), *GIS1* (transcription factor involved in the expression of genes during nutrient limitation), *MUB1* (required for ubiquitylation and degradation of Rpn4p, which stimulates expression of proteasome genes in response to various stress signals), *FAP1* (confers rapamycin resistance by competing with rapamycin for Fpr1p binding), PLM2 (induced in response to DNA damaging agents and deletion of telomerase) and the zinc-finger protein *LEE1*. Several of these factors are induced by cellular stresses or involved in the regulation of carbon metabolism along with the cellular stress response and present attractive targets for further investigation.

Genes enriched for in cluster B under the category metabolic process (GO:0008152; p-value, 0.003652) contain three genes associated with the pentose phosphate pathway (*TKL2*, GND2, XYL2). Genes involved in the pentose phosphate pathway have been shown to be required for resistance to the superoxide generating agent, menadione (Thorpe *et al*, 2004). Interestingly, cluster B also contains the cytochrome c heme lyase, Cyc3p, which has been shown to be required for carnitine's protective function against

H_2O_2 (Chapter 4). Two genes required for cytochrome c oxidase function (*COX20* and *COX23*) that are also part of this cluster, are also upregulated by carnitine in an oxidative stress specific manner. The enhanced expression of these three proteins could be expected to have an impact on the terminal end of the electron transport chain that reduces O^{2-} formed by oxidative phosphorylation (Herrero *et al*, 2008).

The set of genes downregulated by carnitine supplementation during oxidative stress include several functional categories associated with amino acid and purine biosynthesis, namely arginine biosynthesis (MIPS: 01.01.03.05.01; p-value, 1.89e-08), purine nucleotide metabolism (MIPS:01.03.01.03; p-value, 3.305e-05), aromatic compound metabolic process (GO:0006725; p-value, 3.016e-11) and amino acid biosynthetic process (GO:0008652; p-value, 1.29e-08). These genes are mostly grouped in cluster D along with genes involved in cell cycle regulation (GO:0007049; p-value, 0.0004786) and organic acid metabolism (GO:0006082; p-value, 0.0001181). The expression patterns of genes within this cluster are characteristically downregulated upon exposure to H_2O_2 and see a further slight decrease in transcription in the presence of carnitine. The overall pattern, however, suggests the observed expression decrease to be as a result of the impact of H_2O_2 on the cell, which would expectedly lead to cell cycle arrest and also reduction in energy consuming biosynthesis pathways in order to direct more cellular energy towards stress related defences.

Genes involved in mating and sexual reproduction (GO:0019953; p-value, 3.71e-07) were also enriched among transcripts downregulated by carnitine during oxidative stress (Table 5.2). This category includes several genes regulated by the pheromone responsive MAPK pathway (*FUS1*, *KAR4*, *SPR3*, *PRM2*, *PRM10*, *PRM6, SCW10*, *PRM3).* This signaling network has been shown to have an impact on the regulation of apoptosis and in combination with the high osmolarity glycerol (HOG) pathway regulates gene expression of genes in response to environmental stresses, including exposure to various oxidants (Zhang *et al*, 2006; Stavela *et al*, 2004).

The expression of a group of iron-sulfer cluster binding proteins is also downregulated by carnitine (Table 5.2). This groups includes both mitochondrial aconitases (*ACO1* and *ACO2*), which is required for the tricarboxylic acid (TCA) cycle and also independently required for mitochondrial genome maintenance (Chen *et al*, 2005). Aconitases catalyze the interconversion of citrate and isocitrate, and aconitase activities are affected by iron levels, oxidative stress and by the status of the Fe–S cluster biogenesis apparatus (for review see Tong and Rouault, 2007). Additional

mitochondrial iron metabolism genes represented in this group includes *NFU1* (involved in iron metabolism in mitochondria), *ISA1* (involved in biogenesis of the iron-sulfur (Fe/S) cluster of Fe/S proteins) and *ISU1* (performs a scaffolding function during assembly of iron-sulfur clusters, interacts physically and functionally with yeast frataxin, Yfh1p).

Cluster E was enriched specifically for genes that are involved in ribosomal and RNA regulation, which includes the categories of ribosome biogenesis (GO:0042254; p-value, 2.108e-0), RNA modification(GO:0009451; p-value, 7.992e-05), rRNA metabolic process (GO:0016072; p-value, 0.0001021) and transcription from RNA polymerase I promoter (GO:0006360; p-value, 0.006239). Genes in this cluster are upregulated by carnitine addition in both wild type and peroxide treated cultures, while H_2O_2 exposure downregulated expression compared to wild type. Genes of specific interest within these enriched categories are the *TOR1* kinase, which functions as a subunit of TORC1, a complex that controls growth in response to nutrients by regulating translation, transcription, ribosome biogenesis, nutrient transport and autophagy (Wullschleger *et al*, 2006). The regulatory effects of the TORC1 complex have also been indicated to influence life-span by regulating mitochondrial metabolism (Scheike and Finkel, 2007). TORC1 complex mediated regulation has been shown to require the activity of the Sch9p protein kinase (Urban *et al*, 2007). Interestingly, transcription of *SCH9* is also upregulated by carnitine supplementation and follows a similar expression pattern to that of *TOR1* (data not shown). Furthermore, the two transcription factors *AFT2* and *MOT3* also group in cluster E. *AFT2*, has been previously discussed for its role in the regulation of iron homeostasis. The Mot3p transcription factor is involved in repression of a subset of hypoxic genes by Rox1p, as well as of several DAN/TIR genes during aerobic growth and repression of ergosterol biosynthetic genes (Grishin *et al*, 1998; Sertil *et al*, 2003).

Enhanced expression of *TOR1* and *SCH9* is, however, generally associated with a decrease in stress resistance and an increase in apoptosis and both chronological (CLS) replicative life-span (RLS). Furthermore, deletion of *RAS1*, which is also upregulated by carnitine, results in an extension of RLS, while slightly decreasing CLS. In the case of *TOR1*, this has been shown to be caused by the inhibition of the transcription factors *MSN2* and *MSN4*, which are required for the activation of the general stress. Deletion of *SCH9*, has been shown to result in chronological life-span extension independent of *MSN2*/*MSN4*, but requires the activation of the *RIM15* kinase

for activation of stress response genes (reviewed in Kaeberlein *et al*, 2007). The increased expression of these kinases could account for some of the differential expression patterns observed for carnitine supplementation, such as the increase in reserve carbohydrate metabolism, the effects on carbon metabolism, the increase in ribosomal biogeneis and the decrease in autophagy.

This could provide an interesting explanation for the observed effect of carnitine in combination with various redox stress inducing agents (Chapter 5). Since, carnitine was shown to only protect specifically against H_2O_2 and menadione induced oxidative stresses and not exert general protection aganist oxidative stressors, it seems likely that the effect of carnitine is not due to an activation of general oxidative stress responses, but could be accounted for by the effect of carnitine on iron homeostasis and mitochondrial metabolism (which should aleviate the effects of H_2O_2 and superoxide generation by menadione) and possbly also activation of pentose phosphate assoctiated genes (which would specifically be required for menadione resistance). Exposure of cells to thiol modifying agents (DTT and diamide), would be expected to disrupt iron homeostasis by targeting iron-sulfur cluster proteins. The inhibition of general stress associated pathways associated with enhanced *TOR1* and *SCH9* expression, would in such a scenario leave the cell with diminished defenses against the stresses caused by these compounds. A further investigation of the interplay between the three kinases and also the requirement of iron homeostasis and mitochondrial metabolism in carnitine associated phenotypes, would not only enhance understanding of carnitine's effect on cellular physiology, but also further the understanding of the functioning of the regulation of stress metabolism in general.

5.3.3. USING PATHWAY PROJECTIONS TO IDENTIFY CO-ORDINATELY REGULATED TRANSCRIPTS

In a different approach to address the possible involvement of putatively carnitine specific regulated factors, all the genes that are co-ordinately over or under expressed by carnitine supplementation in both wild type and H_2O_2 treated cultures were identified (Figure 5.2). This resulted in the recognition of 41 induced and 20 transcripts that were co-ordinately downregulated. Only three transcription factors are included within the 61 differentially expressed genes, namely *YOX1*, *GAT2* and *MGA1*. Extracting all genes that are potentially up or down regulated (above or below a 1.5 fold change by carnitine in the presence of H_2O_2) by these three transcription factors yields the interaction
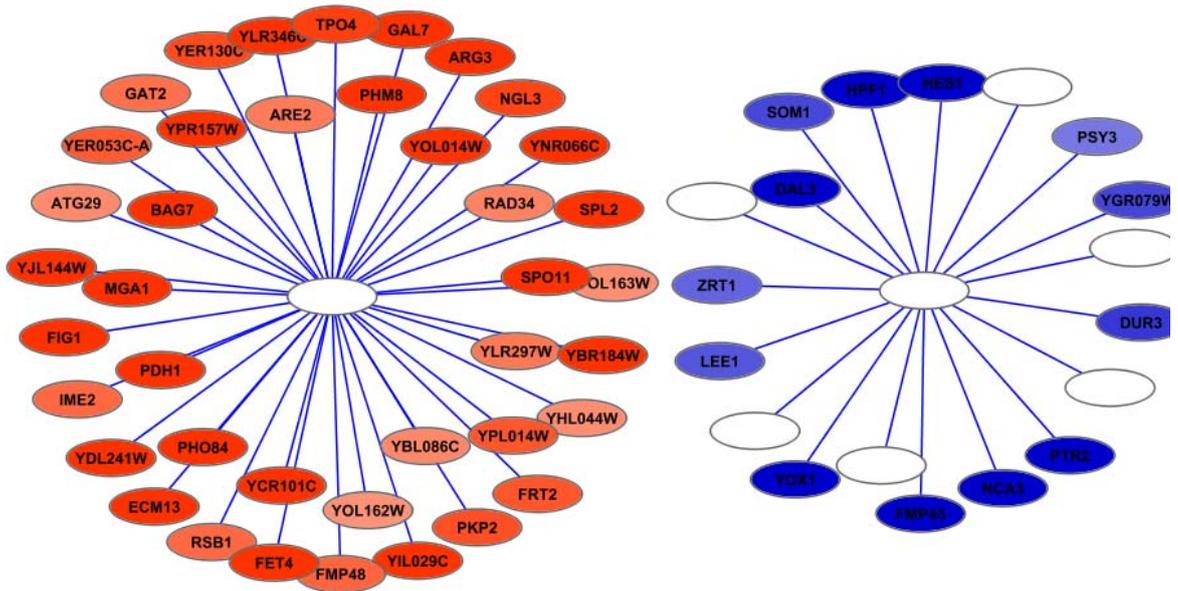
Figure 5.2. Genes that are co-ordinately over or under expressed (using a 1.5 fold cut-of) by carnitine supplementation in both wild type peroxide treated yeast cultures. Red colouring indicates induction and blue indicates transcriptional repression.
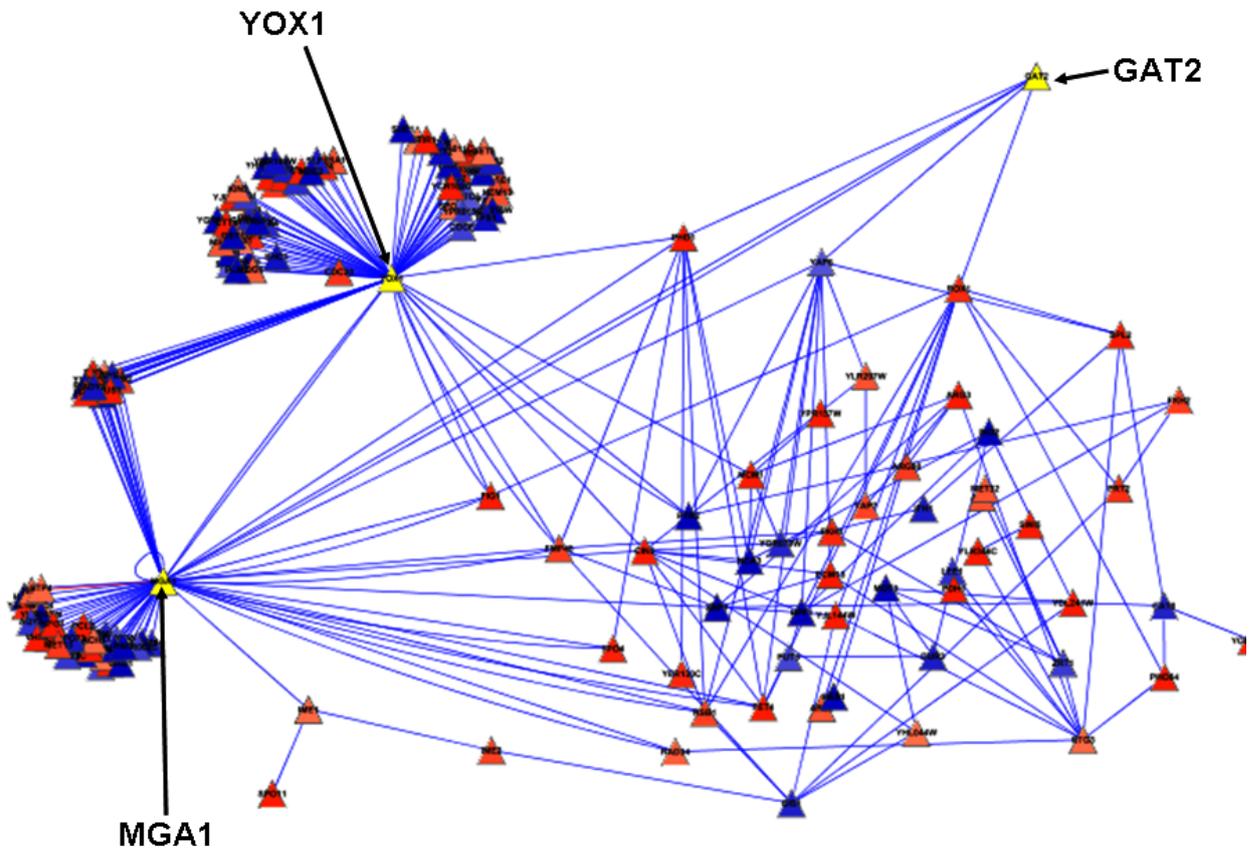
Figure 5.3. The three transcription factors that are co-ordinately regulated by carnitine supplementation to wild type and H_2O_2 treated yeast cultures and their associated genes. Red colouring indicates induction and blue indicates transcriptional repression.

network represented in Figure 5.3. It is strikingly visible that any one of the three transcription factors directly or inderectly regulates the activity of the other two. Furthermore, out of the 155 genes that are affected by *YOX1*, *GAT2* and *MGA1*, 29 transcription factors are represented that include a large amount of the transcription factors that are regulated by carnitine, with or without oxidative stress. This includes the following factors: *RTG3*, *GIS1*, *INO2*, *SWI5*, *MET32*, *YAP6*, *PLM2*, *MIG2*, *MGA1*, *XBP1*, *FKH1*, *SIP4*, *IME1*, *PUT3*, *PHD1*, *IFH1*, *YOX1*, *ARG80*, *MCM1*, *MOT3*, *GAT2*, *CAT8*, *FKH2, YAP7, CIN5*, *MSA1*, *SFG1*, *AFT2* and *ROX1*. This is suggestive of a central role for these three transcription factor in the regulation of carnitine associated effects under condiions of oxidative stress.

5.3.4. USING PATHWAY PROJECTIONS TO INVESTIGATE POSSIBLE LINKS TO APOPTOTIC PATHWAYS
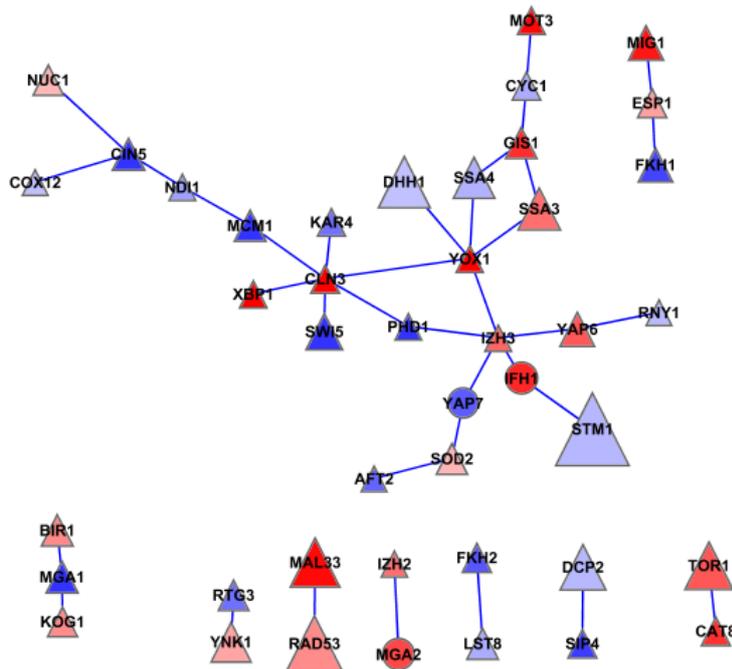
Figure 5.4. Apoptosis related genes are indicated along with regulatory factors. Red colouring indicates induction and blue indicates transcriptional repression.

Exposing yeast cultures to H_2O_2 has been indicated to result in cell death by inducing apoptosis (Madeo *et al*, 1999). Furthermore, carnitine administration has been shown to regulate apoptotic processes in higher eukaryotic organisms (Moretti *et al*, 1998; Mutomba *et al*, 2000; Pastorino *et al*, 1993). In addition, carnitine has been indicated to enhance growth of cultures exposed to H_2O_2 and also drastically diminishes ROS

formation, which serves as a primary trigger for apoptotic induction of H_2O_2 treated cultures (Franken *et al*, 2008; Chapter 4). For these reasons, this work aimed to investigate the effect of carnitine supplementation in yeast cultures on the expression of apoptosis associated genes after exposure to peroxide stress. As a data mining approach, pathway projections were used in order to establish potential connections to apoptosis related factors. Apart from establishing links between factors that are differentially expressed by carnitine supplementation, this approach adds biological significance to lesser expressed transcripts, by placement within the context of significantly expressed regulators.

An extensive search of the *Saccharomyces* genome database (SGD; http://www.yeastgenome.org/) resulted in the identification of 50 genes that are associated with apoptotic phenotypes. Projecting these genes onto the yeast transcription factor network and filtering out all genes that are not differentially expressed (using a fold cut-of of 1.5 for values obtained from H_2O_2 treated *vs* H_2O_2 treated, carnitine supplemented microarray experiments) extracted a total of 22 transcription factors that could potentially be associated with apoptosis (Figure 5.2). For the purpose of this discussion expression changes are indicated as fold change values in brackets, where positive values are upregulated and negative downregulated.

BIR1 (Baculovirus inhibitor-of-apoptosis repeat containing protein), has been indicated to inhibit apoptosis when overexpressed and deletion results in an increase of apoptosis (Li *et al*, 200). Expression of *BIR1* is upregulated by carnitine during oxidative stress (1.25) and is connected to the transcription factor *MGA1* (5.26; similar to heat shock transcription factors), which is regulated by *KOG1* (-1.24; a subunit of the TORC1 complex). *RAD53* encodes a protein kinase, required for cell-cycle arrest in response to DNA damage that has been implicated in the regulation of apoptosis since a reduction of *RAD53* function was shown to increase the occurrence of apoptosis Walter *et al*, 2006). *RAD53* expression has a 1.25 fold increase in response to carnitine, which is likely to be due to its regulation by the transcriptional activator *MAL33* (1.95). The metal ion binding protein *IZH2* encodes a plasma membrane protein involved in zinc metabolism and osmotin-induced apoptosis. A Δ*izh2* null mutant displays an absence of apoptosis (Morton *et al*, 2007). The expression of this gene sees a fold increase of 1.4 in response to carnitine supplementation and is associated with the transcriptional activator *MGA2* (1.62). The *YNK1*, nucleotide diphosphate kinase, has been suggested to function in a signalling capacity in various species and effects the processes of

development, cell differentiation, proliferation, cell motility, tumor metastasis, and apoptosis (Amutha and Pain, 2003). The expression of *YNK1* is slightly induced (1.1) by carnitine and is linked to the transcription factor encoding gene *RTG3* (-1.5) that forms a complex with Rtg1p, to activate the retrograde (RTG) and TOR pathways (Crespo *et al*, 2002). *DCP2* encodes the catalytic subunit of the Dcp1p-Dcp2p decapping enzyme complex, which removes the 5' cap structure from mRNAs prior to their degradation and is also slightly downregulated by carnitine (-1.1). The decrease of *DCP2* could possibly be accounted for by the reduction in *SIP4* expression (-1.9). *TOR1* and *CAT8* was also isolated in this projection, the possible contributions of these factors have been described in section 5.3.2 of this chapter. The caspase-like cysteine protease encoding gene, *ESP1* (Ciosk *et al*, 1998), is slightly upregulated (1.1) in the tripartite cluster regulated by the effects of the transcription factors *MIG1* (1.87) and *FKH1* (-1.8). The *ESP1* null mutant is inviable.

In addition, a central cluster of genes involved in the regulation of apoptosis that is co-regulated by the activities of several shared transcription factors. Significant, among this group of genes is the cyclin *CLN3*, which is upregulated by 2.1 fold by the presence of carnitine. *CLN3* is transcriptionally controlled by the factors *KAR4* (-1.5), *SWI5* (-4.7), *XBP1* (2.1), *YOX1* (2.2), *MCM1* (-2.1) and *PHD1* (2.3). *CLN3* deletion has been shown to result in an increase in apoptosis (Weinberger *et al*, 2007). *PHD1* and *YOX1*, along with the factors *YAP6* (1.5), *YAP7* (-1.7) and *IHF1* (1.8), also co-regulates the expression of *IZH1* (1.45) which encodes a protein involved in zinc metabolism of which a null mutation results in the absence of apoptosis (Morton *et al*, 2007). *YOX1* regulates the transcription of *DHH1* (1.0), *SSA4* (1.0) and *SSA3* (1.4) together with GIS1 (1.7). *SSA3* and *SSA4* encode chaperone proteins that comprise the S. cerevisiae SSA subfamily of cytosolic HSP70 proteins (Werner-Washburne *et al*, 1987). However, the expression of only *SSA3* is significantly increased within this group. *SSA3* encodes an ATPase involved in protein folding and the response to stress and has been shown to protect *S. cerevisiae* cells that express human alpha-synuclein, the protein that forms amyloid fibres in Parkinson disease, from apoptosis (Flower *et al*, 2005). Deletion of *DHH1*, which encodes a cytoplasmic DExD/H-box helicase that stimulates mRNA decapping, has been shown to result in an increase in apoptosis (Mazzoni *et al*, 2003). *GIS1*, in combination with *MOT3* (1.96), regulates the expression of *CYC1*, encoding one of the two yeast cyrochrome c's, which is only slightly induced (1.16). *NDI1* (1.23), which encodes a NADH:ubiquinone oxidoreductase that transfers electrons from NADH

to ubiquinone in the respiratory chain, is induced by the activities of *MCM1* (-2.0) and *CIN5*/*YAP4* (-2.4). Deletion of *NDI1* has been indicated to result in decreased apoptosis (Liang and Zhou, 2007). *CIN5*/*YAP4*, which is an transcription factor of the yAP-1 family, additionally regulates the expression of the genes *NUC1* (1.0; major mitochondrial nuclease of which overexpression increases apoptosis, while deletion reduces apoptotic death) and COX12 (1.0; subunit of cytochrome c oxidase, null mutant decreases apoptosis) (Liang and Zhou, 2007; Buttner *et al*, 2007).

Seeing that 22 of the differentially expressed transcription factors regulate genes involved in apoptotis processes, the involvement of programmed cell death in the observed phenotypes associated with carnitine supplemented cultures under peroxide stress needs to be considered. It is, however, possible that these processes are indirectly affected by the removal of the stress (such as ROS) that would result in apoptotic pathway induction. Further phenotypic analysis of the effect of carnitine on programmed cell death is required to answer these questions. The identified factors present an atrractive starting point for further investigation.

5.4. REFERENCES

Amutha, B. and Pain, D. (2003). Nucleoside diphosphate kinase of *Saccharomyces cerevisiae*, Ynk1p: localization to the mitochondrial intermembrane space. *Biochem J* **370,** 805-15.

Blaiseau, P. L., Lesuisse, E. and Camadro, J. M. (2001). Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. *J Biol Chem* **276,** 34221-6.

Bremer, J. (1983). Carnitine-metabolism and functions. *Physiol Rev* **63,** 1420-80.

Buttner, S., Eisenberg, T., Carmona-Gutierrez, D., Ruli, D., Knauer, H., Ruckenstuhl, C., Sigrist, C., Wissing, S., Kollroser, M., Frohlich, K. U., Sigrist, S. and Madeo, F. (2007). Endonuclease G regulates budding yeast life and death. *Mol Cell* **25,** 233-46.

Calabrese, V., Giuffrida Stella, A. M., Calvani, M. and Butterfield, D. A. (2006). Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes. *J Nutr Biochem* **17,** 73-88.

Chen, X. J., Wang, X., Kaufman, B. A. and Butow, R. A. (2005). Aconitase couples metabolic regulation to mitochondrial DNA maintenance. *Science* **307,** 714-7.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998). An *ESP1*/*PDS1* complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93,** 1067-76.

Cohen, B. D., Sertil, O., Abramova, N. E., Davies, K. J. and Lowry, C. V. (2001). Induction and repression of *DAN1* and the family of anaerobic mannoprotein genes in *Saccharomyces cerevisiae* occurs through a complex array of regulatory sites. *Nucleic Acids Res* **29,** 799-808.

Crespo, J. L., Powers, T., Fowler, B. and Hall, M. N. (2002). The TOR-controlled transcription activators

GLN3, *RTG1*, and *RTG3* are regulated in response to intracellular levels of glutamine. *Proc Natl Acad Sci U S A* **99,** 6784-9.

Eisen, M. B., Spellman, P. T., Brown, P. O. and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* **95,** 14863-8.

Flower, T. R., Chesnokova, L. S., Froelich, C. A., Dixon, C. and Witt, S. N. (2005). Heat shock prevents alpha-synuclein-induced apoptosis in a yeast model of Parkinson's disease. *J Mol Biol* **351,** 1081-100.

Franken, J., Kroppenstedt, S., Swiegers, J. H. and Bauer, F. F. (2008). Carnitine and carnitine acetyltransferases in the yeast *Saccharomyces cerevisiae*: a role for carnitine in stress protection. *Curr Genet* **53,** 347-60.

Grishin, A. V., Rothenberg, M., Downs, M. A. and Blumer, K. J. (1998). Mot3, a Zn finger transcription factor that modulates gene expression and attenuates mating pheromone signaling in *Saccharomyces cerevisiae*. *Genetics* **149,** 879-92.

Herrero, E., Ros, J., Belli, G. and Cabiscol, E. (2008). Redox control and oxidative stress in yeast cells. *Biochim Biophys Acta* **1780,** 1217-35.

Kim, J. H. and Powers, S. (1991). Overexpression of *RPI1*, a novel inhibitor of the yeast Ras-cyclic AMP pathway, down-regulates normal but not mutationally activated ras function. *Mol Cell Biol* **11,** 3894-904.

Kowalski, L. R., Kondo, K. and Inouye, M. (1995). Cold-shock induction of a family of TIP1-related proteins associated with the membrane in *Saccharomyces cerevisiae*. *Mol Microbiol* **15,** 341-53.

Larsson, C., Pahlman, I. L., Ansell, R., Rigoulet, M., Adler, L. and Gustafsson, L. (1998). The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*. *Yeast* **14,** 347-57.

Li, F., Flanary, P. L., Altieri, D. C. and Dohlman, H. G. (2000). Cell division regulation by *BIR1*, a member of the inhibitor of apoptosis family in yeast. *J Biol Chem* **275,** 6707-11.

Liang, Q. and Zhou, B. (2007). Copper and manganese induce yeast apoptosis via different pathways. *Mol Biol Cell* **18,** 4741-9.

Mazzoni, C., Mancini, P., Verdone, L., Madeo, F., Serafini, A., Herker, E. and Falcone, C. (2003). A truncated form of KlLsm4p and the absence of factors involved in mRNA decapping trigger apoptosis in yeast. *Mol Biol Cell* **14,** 721-9.

Robinson, M. D., Grigull, J., Mohammad, N. and Hughes, T. R. (2002). FunSpec: a web-based cluster interpreter for yeast. *BMC Bioinformatics* **3,** 35.

Rodrigues-Pousada, C. A., Nevitt, T., Menezes, R., Azevedo, D., Pereira, J. and Amaral, C. (2004). Yeast activator proteins and stress response: an overview. *FEBS Lett* **567,** 80-5.

Schieke, S. M. and Finkel, T. (2007). TOR and aging: less is more. *Cell Metab* **5,** 233-5.

Schmitt, M. E., Brown, T. A. and Trumpower, B. L. (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res* **18,** 3091-2.

Sertil, O., Kapoor, R., Cohen, B. D., Abramova, N. and Lowry, C. V. (2003). Synergistic repression of anaerobic genes by Mot3 and Rox1 in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **31,** 5831-7.

Staleva, L., Hall, A. and Orlow, S. J. (2004). Oxidative stress activates *FUS1* and *RLM1* transcription in the yeast *Saccharomyces cerevisiae* in an oxidant-dependent manner. *Mol Biol Cell* **15,** 5574-82.

Stella, C.A., Burgos, H.I., Salellas, M.L., Cristaldo, M.L., Ramos, E.H., Kriguer, N. (2005). L-Carnitine

effect upon iron growth inhibition on *Saccharomyces cerevisiae*. *Letters in Drug Design and Discovery* **2,** 44-47.

Takagi, H. (2008). Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. *Appl Microbiol Biotechnol* **81,** 211-23.

Thorpe, G. W., Fong, C. S., Alic, N., Higgins, V. J. and Dawes, I. W. (2004). Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes. *Proc Natl Acad Sci U S A* **101,** 6564-9.

Tong, W. H. and Rouault, T. A. (2007). Metabolic regulation of citrate and iron by aconitases: role of iron-sulfur cluster biogenesis. *Biometals* **20,** 549-64.

Urban, J., Soulard, A., Huber, A., Lippman, S., Mukhopadhyay, D., Deloche, O., Wanke, V., Anrather, D., Ammerer, G., Riezman, H., Broach, J. R., De Virgilio, C., Hall, M. N. and Loewith, R. (2007). Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*. *Mol Cell* **26,** 663-74.

Walter, D., Wissing, S., Madeo, F. and Fahrenkrog, B. (2006). The inhibitor-of-apoptosis protein Bir1p protects against apoptosis in *S. cerevisiae* and is a substrate for the yeast homologue of Omi/HtrA2. *J Cell Sci* **119,** 1843-51.

Weinberger, M., Feng, L., Paul, A., Smith, D. L., Jr., Hontz, R. D., Smith, J. S., Vujcic, M., Singh, K. K., Huberman, J. A. and Burhans, W. C. (2007). DNA replication stress is a determinant of chronological lifespan in budding yeast. *PLoS One* **2,** e748.

Werner-Washburne, M., Stone, D. E. and Craig, E. A. (1987). Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* **7,** 2568-77.

Wu, Z. and Irizarry, R. A. (2004). Preprocessing of oligonucleotide array data. *Nat Biotechnol* **22,** 656-8; author reply 658.

Wullschleger, S., Loewith, R. and Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell* **124,** 471-84.

Zhang, N. N., Dudgeon, D. D., Paliwal, S., Levchenko, A., Grote, E. and Cunningham, K. W. (2006). Multiple signaling pathways regulate yeast cell death during the response to mating pheromones. *Mol Biol Cell* **17,** 3409-22.

Chapter 6

RESEARCH RESULTS IV

Reconstruction of the carnitine biosynthesis pathway from *Neurospora crassa* in the brewer's yeast *Saccharomyces cerevisiae*.

ABSTRACT

L-carnitine plays an essential role in eukaryotic energy metabolism by mediating the transfer of activated acyl residues between organellar compartments. In eukaryotes, including the fungus *Neurospora crassa*, L-carnitine is biosynthesized through the stepwise hydroxylation and dehydrogenation of the precursor trimethyllysine. It has, however, been shown that the yeast *Saccharomyces cerevisiae* is unable to neo-synthesize carnitine and is entirely dependent on extracellular supplementation. This study describes the cloning and characterization of all four of the carnitine biosynthesis genes from *N. crassa* and the reconstruction of the pathway in *S. cerevisiae*. In addition the free lysine methyltransferase from *N. crassa*, which converts lysine to trimethyllysine through sequential methylation was also cloned and heterologously expressed in yeast. A preliminary analysis of the engineered strains capacity to produce L-carnitine indicates that a strain bearing the reconstructed carnitine biosynthesis pathway is able to convert trimethyllysine to L-carnitine.

6.1. INTRODUCTION

L-Carnitine functions as a shuttling molecule, which facilitates the transfer of activated acyl residues across the membranes of peroxisomes and mitochondria to enable further metabolism. This function is supported by the activities of various carnitine acyl-transferases and transporters, which are collectively referred to as the carnitine shuttle (reviewed in Ramsey *et al*, 2004). The importance of this compound in energy metabolism became evident in early studies which attributed various diseases to a dysfunction in the carnitine metabolism (reviewed in Longo *et al*, 2006). In mammalian systems, carnitine uptake accounts for about 75% of the systemic requirement, the remaining 25% being provided through endogenous biosynthesis (Tein *et al*, 1996). Higher eukaryotes are able to neosynthesize their own carnitine by means of a four step biosynthesis pathway, which facilitates the enzymatic hydroxylation and dehydrogenation of the pathway's precursor, trimethyllysine. Mutations in carnitine biosynthesis genes can be effectively treated through dietary carnitine supplementation. Furthermore, supplementation of either carnitine or acetylcarnitine has been shown to be of therapeutic benefit in several diseases associated with mitochondrial function (Calabrese *et al*, 2005).

Since the initial description of the eukaryotic carnitine biosynthesis pathway in *Neurospora crassa*, the conserved enzymatic activities and most of the genes involved have been extensively characterised in various higher eukaryotes (reviewed in Vaz and Wanders, 2002; Figure 6.1). The first step, the hydroxylation of trimethyllysine to hydroxyl-trimethyllysine, is catalyzed by the activity of the enzyme trimethyllysine hydroxylase (TMLH), which has been cloned from humans and also from *N. crassa* (Vaz *et al*, 2001; Swiegers *et al*, 2002). This product is aldolytically cleaved by hydroxy-trimethyllysine aldolase (HTMLA) to form the next intermediate, trimethylaminobutyraldehyde. This enzyme has not been cloned from any organism but early studies in rats speculated this activity to be that of a serine hydroxyl methyltransferase (Henderson *et al*, 1982; Stein and Englard, 1981). Trimethylaminobutyraldehyde is further dehydrogenated by the enzyme trimethylaminobutyraldehyde dehydrogenase (TMABA-DH), leading to the formation of the last intermediate, γ-butyrobetaïne. The gene encoding this dehydrogenase has been successfully isolated and characterized in studies on humans and rats (Vaz *et al*, 2000;

Kikonyogo and Pietruszko, 1996; Lin *et al*, 1996; Kurys *et al*, 1993; Chern and Pietruszko, 1995). The final hydroxylation step in this pathway is catalyzed by γ-butyrobetaïne hydroxylase (BBH) to form L-carnitine. The human gene encoding BBH has previously been cloned (Vaz *et al*, 1998). In addition to the central pathway for carnitine biosynthesis, *N. crassa* has been shown to perform stepwise methylations of free lysine in order to form the precursor trimethyllysine (Borum and Broquist, 1977). In higher eukaryotic organisms this compound is mainly sourced from trimethyllysine pools formed as a byproduct of protein degradation (La Badie *et al*, 1976; Dunn *et al*, 1984).
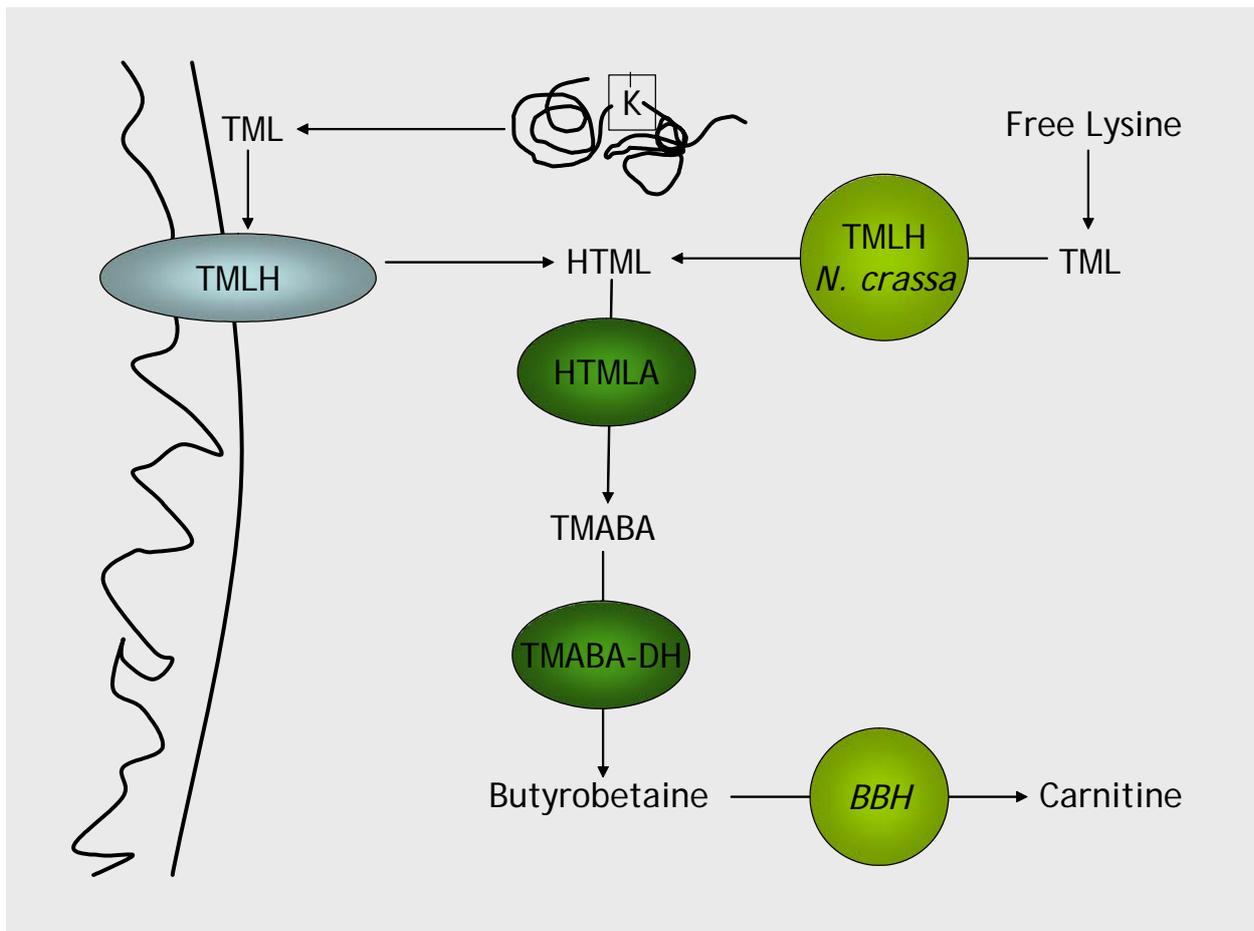
Figure 6.1. Schematic representation of the eukaryotic carnitine biosynthesis pathway.

In the brewer's yeast, *Saccharomyces cerevisiae*, two separate metabolic routes exist in order to channel activated acyl residues to the mitochondrial TCA cycle. Apart from the carnitine shuttle, an alternate pathway, referred to as the glyoxylate-cycle, combines two acetyl-CoA units in order to form succinate, which can freely enter the mitochondria for further metabolism (Van Roermund *et al*, 1995). When this metabolic "bypass" is blocked through deletion of the citrate synthase encoding *CIT2* gene, the first enzyme in the glyoxylate cycle, yeast cells are completely dependent on the

carnitine shuttle and on the presence of carnitine in the environment for growth on non-fermentable carbon sources (Swiegers *et al*, 2001). These results indicate that *S. cerevisiae*, unlike most eukaryotes, is unable to neo-synthesize its own carnitine and is solely dependent on carnitine uptake from the extra-cellular environment.

The beneficial attributes of L-carnitine as a dietary supplement and therapeutic agent, along with the wide range of genetic tools available for the introduction and heterologous expression of genes in the model organism *S. cerevisiae* presents an area of genetic research with a variety of possible industrial applications. This study describes the cloning, characterization and expression of the four genes from the *N. crassa* carnitine biosynthesis pathway in *S. cerevisiae*. Furthermore, the gene coding for the free lysine methyltransferase was also cloned from the same organism. All five genes from *N. crassa* were heterologously co-expressed in a laboratory strain of *S. cerevisiae*, thereby reconstituting the pathway in yeast. The constructed strains were used in a preliminary plate based assay for carnitine production. The results indicated that a yeast strain bearing all four of the central carnitine biosynthesis enzymes is able to synthesize carnitine from trimethyllysine. The assay system used here, however, does not allow ascertaining whether the free lysine methyl transferase is actively expressed in *S. cerevisiae*.

6.2. MATERIALS AND METHODS

6.2.1. MICROBIAL STRAINS AND MEDIA

Escherichia coli carrying plasmids were grown in Luria Bertani (LB) broth with 10 mgL^{-1} ampicillin. Yeast strains were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose), synthetic glucose medium (6.7 gL-1 yeast nitrogen base without amino acids, 2% glucose, amino acids as required) and synthetic ethanol medium (6.7 gL-1 yeast nitrogen base without amino acids, 3% ethanol and amino acids as required). The *N. crassa* wild type strains, 74-OR-1VA (Fungal Genetic Stock Centre) and PPRI3338 (National Collection of Fungi, Agricultural Research Council, Pretoria, South Africa), were grown in potato dextrose media (Sigma) and on Vogel's N medium. Growth and media conditions for *N. crassa* have been previously described (Davis and deSerres 1970; Davis, 2000).

5.2.2 RNA EXTRACTION AND cDNA SYNTHESIS FROM *Neurospora crassa*

Mycelia from growing plate cultures of *N. crassa* were inoculated into 5 ml starter cultures of Vogel's N medium containing 1.5% sucrose. After 2 days growth at 28°C enough biomass was formed for transfer to larger volumes of fresh media. The sphere of fungal mass was removed from the media and slices of similar thickness were transferred to flask containing 100 ml of Vogel's N medium with glucose, as carbon source and grown at 28°C for two days. The spheres of growing fungus were removed from the media and allowed to drain on Whattman paper. The drained fungal mass was flash frozen in liquid nitrogen and grinded with a mortar and pestle to a uniform powder to be kept at -80°C for RNA extractions when required. RNA extraction was performed as previously described (Siebert *et al*, 1993). cDNA was synthesized using the SuperScript® III system from Invitrogen.

Table 6.1. Primers used in this study. Introduced restriction sites are italicized.

Primer	Sequence
NC-TMLH-F	5'-gatc*gaattc*ATGAGACCGCAACGGGTAGGGGCA-3'
Cbs-1 revII_EcoRI	5'-gccc*gaattc*CTTAACCAGTAACCCTCGGAAGAACC-3'
NcSHMT-F	5'-gatc*gaattc*ATGTCGAGCTTCCAGAGCACAG-3'
NcSHMT-R	5'-gatc*gtcgac*CTAAGACGACTGGTCCCAAGGG-3'
NCU03415-F	5'-tata*gaattc*ATGTCTTCCAACGTCTTTGTTG–3'
NCU03415-R	5'–tata*gtcgac*CTAGTTGAGCTTGATAGCAACAGAC-3'
NcBBH-F	5'-gatc*agatct*ATGAAAGTCGACAAGGAAGCCGGCAA-3'
NcBBH-R	5'-gatc*agatct*TTATGCGTTCCAGTTCACCGTGCCCAA-3'
NCU03826_rev	5'- GTCGGCATATG TTAGACGTTGGTCAACTTG-3'
NCU03826_fw	5'- GTAGAATTCCATGGCCTTCGGAAAGCTTTAC-3'

6.2.3. CLONING OF CARNITINE BIOSYNTHESIS GENES FROM *N. crassa*

All primers used in the cloning of the carnitine biosynthesis genes are listed in Table 6.1. Clones and constructs are listed in Table 6.2. The gene encoding for the *N. crassa* trimethyllysine dehydroxylase (NcTMLH) was amplified from *N crassa* cDNA using the primer set NC-TMLH-F (5'-gatcgaattc ATG AGA CCG CAA CGG GTA GGG GCA-3') and Cbs-1 revII_*Eco*RI (5'-gcccgaattc CTT AAC CAG TAA CCC TCG GAA GAA CC-3'). The resulting 1416 bp fragment was cloned into the pGEM-TEasy cloning vector purchased from Promega. For integration and expression in yeast this fragment was

subcloned into the *PGK1* promoter/terminator cassette of the yeast integration vector pRS-305 using the *Eco*RI sites (indicated in italics in the primer sequence) introduced through PCR at the 5' and 3' ends of the fragment. The construct was linearized using *Bst*EII, for integration into the *LEU2* locus, and transformed into the FY23 and also in combination with the other biosynthesis constructs.

For the cloning of the mitochondrial serine hydroxyl methyltransferase (NcSHMT), cDNA from *N. crassa* was used as template together with the primer NcSHMT-F (5'-gatcgaattc ATG TCG AGC TTC CAG AGC ACA G-3') binding at the ATG and NcSHMT-R (5'-gatcgtcgac CTA AGA CGA CTG GTC CCA AGG G-3') binding at the STOP codon of the open reading frame. The amplified fragment was cloned into pGEM-Teasy. A 1584 bp fragment was excised from this construct using the 5' *Eco*RI and 3' *Bgl*II sites introduced through PCR and cloned into the *PGK1* promoter/terminator cassette of the centromeric vector YCpLac22-PGKpt.

The following primers were used for the amplification of the *N. crassa* trimethylaminobutyraldehyde dehydrogenase (NcTMABA-DH) from *N. crassa* cDNA; NCU03415-F (5'- tatagaattc ATG TCT TCC AAC GTC TTT GTT G – 3'), binding at the ATG and NCU03415-R (5' – tatagtcgac CTA GTT GAG CTT GAT AGC AAC AGA C - 3'), binding at the STOP codon of the coding region. The amplified fragment was cloned into the pGEM-Teasy vector. A fragment of 1493 bp was excised using the introduced *Eco*RI and *Bgl*II sites and subcloned using the same restriction sites into the *PGK1* promoter/terminator expression cassette of the pCEL13 integration vector. For integration, the construct was linearized using an *Nco*I site that digests inside the *URA3* marker of pCEL13 and used in yeast transformations of the FY23 wild type strain and also in combination with the other carnitine biosynthesis constructs.

For the cloning of the *N. crassa* butyrobetaïne hydroxylase (NcBBH), a 2016 bp fragment was cloned from *N. crassa* genomic DNA, extracted from strain PPRI 3338, 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 5' and 3' *Bgl*II restriction sites (indicated in italics). The fragment was cloned into the cloning vector pGEM-Teasy, from where it was subcloned into the *Bgl*II site of the integration vector pSP-*PGK1*pt. The cloned fragment is under the regulation of the *PGK1* promoter and terminator. The construct was linearized using *Eco*NI, for integration into the *ILV2* locus, using sulfometron methyl for selection (Casey *et al*,

1988). The linearized integration cassette was subsequently transformed into the FY23 wild type strain and also in combination with the other carnitine biosynthesis constructs.

The gene sequence coding for the free lysine methyltransferase (NcFLMT), *N. crassa* ORF NCU03826, was amplified using the primer set NCU03826_rev and NCU03826_fw and cloned into pGEM-Teasy. The fragment containing the free lysine methyltransferase ORF was excised using the introduced 5' *Eco*RI and 3' *Nde*I restriction sites and cloned into the *PGK1* expression cassette of pDMPL. The cassette containing the *PGK1* promoter, FLMT open reading frame and *PGK1* terminator was excised using the *Bbr*PI and *Nhe*I restriction sites and cloned into pPOF using the same restriction sites. The plasmid was linearized using *Sma*I for transformation into the *POF1* locus and the KanMX marker (geneticin resistance) present on the cassette was used for selection. The FLMT integration construct was transformed into the FY23 wild type strain and also into the FY23 strain containing all four carnitine biosynthesis genes. The generated strains are listed in Table 6.3. All clones were sequenced using the ABI-Prism sequencer to confirm the integrity of PCR amplifications. Integration of constructs was confirmed using a combination of primers that binds the integrated cassette and also the genomic DNA adjacent to the sites of integration for PCR amplifications.

Table 6.2. Clones and constructs

Plasmids	Relevant genotype	Sources and references
YCpLac22-PGK1pt	CEN4 *TRP1* PGK1*p*-PGK1*t*	This laboratory
YCpLac22-PGK1pt-HTMLA	CEN4 *TRP1* PGK1*p*-HTMLA-PGK1*t*	This study
pRS305	CEN6 LEU2 PGK1*p*-PGK1*t*	Sikorski and Hieter,1989
pRS305-TMLH	CEN6 *LEU2* PGK1*p*-TMLH -PGK1*t*	This study
pSP-*PGK1*pt	SMR PGK1*p*-PGK1*t*	Sunbio
pSP-*PGK1*pt-BBH	SMR PGK1*p*-BBH-PGK1*t*	This study
pCEL13	2μ *URA3* PGK1*p*-PGK1*t*	Moses *et al*, 2005
pCEL13-TMABA-DH	2μ *URA3* TMABA-DH	This study
pGEM-Teasy		Promega.
pDMPL	*PGK1*p-*PGK1*t	SunBio
pDMPL-FLMT	*PGK1*p-FLMT-*PGK1*t	SunBio
pPOF-	*KanMX4*	SunBio
pPOF-FLMT	*KanMX4 PGK1*p-FLMT-*PGK1*t	This study

6.2.4 CARNITINE PRODUCTION SCREEN USING A Δ*cit2 YEAST* STRAIN

The screen for carnitine production is based on the carnitine-dependent phenotype of the Δ*cit2* deletion strain. A liquid culture of Δ*cit2* was grown up overnight in SCD media, without carnitine. The cultures were harvested and after thorough washing, plated as a mat on a synthetic agar medium containing a non-fermentable carbon source, ethanol, and no carnitine. Growing yeast colonies that biosynthesise carnitine produce a zone of growth due to the complementation of the Δ*cit2* mutant by carnitine made available from the producing strain.

Table 6.3. Yeast strains used in this study

Yeast strains	Relevant genotype	Sources and references
FY23	*MATa leu2 trp1 ura3*	Winston *et al.* 1995
FY23 BBH	*MATa leu2 trp1 ura3 BBH*	This study
F23 TMLH BBH	*MATa leu2 trp1 ura3 TMLH BBH*	This study
FY23 TMLH HTMLA BBH	*MATa leu2 trp1 ura3 TMLH HTMLHA BBH*	This study
FY23 TMLH TMABA-DH BBH	*MATa leu2 trp1 ura3 TMLH TMABA-DH BBH*	This study
FY23 TMLH HTMLA TMABA-DH BBH	*MATa leu2 trp1 ura3 TMLH HTMLA TMABA-DH BBH*	This study
FY23 CB	*MATa leu2 trp1 ura3 FLMT TMLH HTMLA TMABA-DH BBH*	This study

6.3. RESULTS

6.3.1. CLONING AND CHARACTERISATION OF THE *N. crassa* FREE LYSINE METHYLTRANSFERASE (NcFLMT)

The cloning and expression of the *N. crassa* free lysine methyltransferase (*N. crassa* ORF NCU03826) in *E. coli* was previously described in an international patent application (application No. WO 2007/007987 A1). Primers for the amplification of *N. crassa* ORF NCU03826 (EMBL accession No. Q7S7R7) were designed according to the sequence submitted in the application. The predicted sequence of the gene encodes and ORF of 1059 bp. The coding region includes 5' and 3' UTR's of 33 and

2002). The predicted sequence contains 6 exons and codes for a 1978 bp gene, with a resulting protein product of 528 amino acids. Furthermore, a 3' untranslated region of 229 bp is present which was confirmed by Swiegers *et al* (2002). For the purposes of this work it was decided to re-isolate this gene from cDNA with a primer set that amplifies the coding region without the UTR. A fragment of 1416 bp, coding for a predicted protein product of 472 amino acids with a molecular weight of 52.7 kDa (EMBL accesion No. AJ421151) was amplified. The cloned TMLH gene from *N. crassa* shares significant similarity to the gene sequences that have been previously isolated from rat and human and also to the sequence of γ-butyrobetaïne hydroxylase (Swiegers *et al*, 2002; Figure 6.2.).

```
              1                                                  50
NcSHMT    (1) MSTYSLSETHKAMLEHSLVESDPQVAEIMKKEVQRQRESIILIASENVTS
ScSHMT2   (1) -MPYTLSDAHHKLITSHLVDTDPEVDSIIKDEIERQKHSIDLIASENFTS
ratSHMT   (1) --------------------------------------------------
Consensus (1)    YSLSD H  LI   LVDSDP V  IIK EI RQK SI LIASEN TS
              51                                                100
NcSHMT   (51) RAVFDALGSPMSNKYSEGLPGARYYGGNQHIDEIEVLCQNRALEAFHLDP
ScSHMT2  (50) TSVFDALGTPLSNKYSEGYPGARYYGGNEHIDRMEILCQQRALKAFHVTP
ratSHMT   (1) --------------------------------------------------
Consensus (51)  AVFDALGSPLSNKYSEG PGARYYGGN HID IEILCQNRAL AFHL P
              101                                               150
NcSHMT  (101) KQWGVNVQCLSGSPANLQVYQAIMPVHGRLMGLDLPHGGHLSHGYQTPQR
ScSHMT2 (100) DKWGVNVQTLSGSPANLQVYQAIMKPHERLMGLYLPDGGHLSHGYATENR
ratSHMT   (1) -------------------------PSENGDSLPLFGPLLLGGLCPAR
Consensus (101)   WGVNVQ LSGSPANLQVYQAIM  H RLMGL LP GGHLSHGY TPNR
              151                                               200
NcSHMT  (151) K---ISAVSTYFETMPYRVNIDTGLIDYDTLEKNAQLFRPKVLVAGTSAY
ScSHMT2 (150) K---ISAVSTYFESFPYRVNPETGIIDYDTLEKNAILYRPKVLVAGTSAY
ratSHMT  (24) RSAGLAGLRTLLGGLSLLVYPDTGYINYDQLEENASLFHPKLIIAGTSCY
Consensus (151) K   ISAVSTYFESLPYRVNPDTGIIDYDTLEKNA LFRPKVLVAGTSAY
              201                                               250
NcSHMT  (198) CRLIDYERMRKIADSVGAYLVVDMAHISGLIASEVIPSPFLYADVVTTTT
ScSHMT2 (197) CRLIDYKRMREIADKCGAYLMVDMAHISGLIAAGVIPSPFEYADIVTTTT
ratSHMT  (74) SRNLDYARLRKIADDNGAYLMADMAHISGLVAAGVVPSPFEHCHVVTTTT
Consensus (201) CRLIDY RMRKIAD  GAYLMVDMAHISGLIAAGVIPSPFEYADVVTTTT
              251                                               300
NcSHMT  (248) HKSLRGPRGAMIFFRRGVRSVDAKTGKETLYDLEDKINFSVFPGHQGGPH
ScSHMT2 (247) HKSLRGPRGAMIFFRRGVRSINPKTGKEVLYDLENPINFSVFPGHQGGPH
ratSHMT (124) HKTLRGCRAGMIFYRKGVRSVDPKTGEETYYELESLINSAVFPGLQGGPH
Consensus (251) HKSLRGPRGAMIFFRRGVRSVDPKTGKETLYDLE  INFSVFPGHQGGPH
              301                                               350
NcSHMT  (298) NHTITALAVALKQAASPEFKEYQQKVVANAKALEKKLKELGYKLVSDGTD
ScSHMT2 (297) NHTIAALATALKQAATPEFKEYQTQVLKNAKALESEFKNLGYRLVSNGTD
ratSHMT (174) NHAIAGVAVALKQAMTTEFKIYQLQVLANCRALSDALTELGYKIVTGGSD
Consensus (301) NHTIAALAVALKQAATPEFKEYQ QVLANAKALE  LKELGYKLVS GTD
              351                                               400
NcSHMT  (348) SHMVLVDLRPIGVDGARVEFLLEQINITCNKNAVPGDKSALTPGGLRIGT
ScSHMT2 (347) SHMVLVSLREKGVDGARVEYICEKINIALNKNSIPGDKSALVPGGVRIGA
ratSHMT (224) NHLILMDLRPKGTDGGRAEKVLEACSIACNKNTCPGDKSALRPSGLRLGT
Consensus (351) SHMVLVDLRPKGVDGARVEFILE INIACNKNSIPGDKSAL PGGLRIGT
              401                                               450
NcSHMT  (398) PAMTSRGFGEADFEKVAVFVDEAVKLCKEIQASLPKEANKQKDFKAKIAT
ScSHMT2 (397) PAMTTRGMGEEDFHRIVQYINKAVEFAQQVQQSLPKDACRLKDFKAKVDE
ratSHMT (274) PALTSRGLLEEDFQKIAHFIHRGIELTLQIQSHMTMRAT-LKEFKEKLTG
Consensus (401) PAMTSRGLGEEDF KIA FI KAVEL  QIQASLPKDA KLKDFKAKI
              451                                486
NcSHMT  (448) SDIPR--TNELKQEIAAWSNTFPLPVEGWRYDAGL-
ScSHMT2 (447) GSDVLN---TWKKEIYDWAGEYPLAV----------
ratSHMT (323) DEKFQSAVAALREEVENFASNFSLPGLPDF------
Consensus (451) D      I  LK EI  WA  FPLPV
```

Figure 6.3. Alignment of the cloned serine hydroxymethyltransferase (SHMT) from *N. crassa* to the rat serine hydroxy-methyltransferase gene and also the *S. cerevisiae* Shm2p.

6.3.3. CLONING AND CHARACTERISATION OF THE *N. crassa* HYDROXY TRIMETHYLLYSINE ALDOLASE (NcSHMT)

A previous study reported the isolation of a serine hydroxymethyltransferase (SHMT) from rat liver that was able to catalyze the conversion of hydroxytrimethyllysine to trimethylaminobutyraldehyde, the second step of carnitine biosynthesis (Henderson *et al* 1980; 1982). A search of the *Neurospora* genome database (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html) identified two SHMT's in the fungal genome, one coding for a cytosolic and the other a mitochondrial enzyme. It was decided to clone the cytosolic isoform (NCU02274; EMBL accession No. P34898), since the rest of the carnitine biosynthesis activities are located in the cytosol. The predicted locus from the Neurospora genome database consists of an open reading frame spanning 2248 bp, containing 3 exons and leads to a processed product of 2097 bp. The gene also contains 5' and 3' UTRs of 99 and 558 bp respectively. The primer set designed for the isolation of SHMT amplifies the central region, excluding both UTRs, and resulted in an amplicon of 1443bp. The sequence of the cloned SHMT does not differ from the predicted sequence of the Neurospora Genome Database. The predicted protein product contains 481 amino acids and has a molecular weight of ~ 53 kDA. The *N. crassa* SHMT shares 73% homology to the rat SHMT gene (EMBL accession No. BC099219*)* and 80% homology to that of *S. cerevisiae* Shm2p (Figure 6.3.).

6.3.4. CLONING AND CHARACTERISATION OF THE *N. crassa* TRIMETHYLAMINOBUTYRALDEHYDE DEHYDROGENASE (NcTMABA-DH)

A gene family of aldehyde dehydrogenases was identified using the sequence of the human and rat trimethylaminobutyraldehyde dehydrogenases in a BLAST analysis of the *N. crassa* genome. Of these enzymes the enzyme under the accession number NCU03415 (EMBL accession No. Q8X0L4) had the closest similarity (~60%) to the previously identified rat and human copies. The predicted genomic sequence contains 3 exons coding for a primary transcript of 1853bp with 5' and 3' UTRs of 139 and 211 bp respectively. This would lead to a predicted peptide consisting of 495 amino acids with a molecular mass of ~ 54 kDa.

A fragment of 1488 bp was amplified from cDNA using primers homologous to the 5' and 3' regions of the gene NCU03415, which excludes the respective UTRs. The sequence of the cloned fragment is 100% similar to that of the *N. crassa* gene

NCU03415, as indicated in the *N crassa* genome database. The cloned NcTMABA-DH shares 60% similarity to that of the previously cloned ALDH9 gene from rat, which has been indicated to function as a trimethyl-aminbutyraldehyde dehydrogenase (Lin *et al*, 1996; Figure 6.4.). Furthermore, this gene also has significant homology to the four aldehyde dehydrogenase enzymes present in the S288c genetic background, namely Ald4p (67%), Ald2p (66%), Ald5p (65%), and Ald3p (64%).

```
                        1                                                 50
NcTMABA-DH    (1)   MEVELTAPNGKKWMQPLGLFINNEFVKSANEQKLISINPTTEEEICSVYA
ratTMABA-DH   (1)   -----MSTGTFVVSQPLNYRGGARVEPVDASGTEKAFEPATGREIATFKC
 Consensus    (1)        A       QPL                    A  P T  EI S
                        51                                                100
NcTMABA-DH   (51)   ATAEDVDAAVSAARKAFRHESWKSLSGTERGALMRKLADLVAENAEILAT
ratTMABA-DH  (46)   SGEKEVNLAVENAKAAFKIWSKK--SGLERCQVLLEAARIIKERRDEIAI
 Consensus   (51)   A   DV AV  AK AFK  S K   SG ER LL   A II E  D IA
                        101                                               150
NcTMABA-DH  (101)   IECLDNGKPYQTALNENVPEVINVLRYYAGYADKNFGQVIDVGPAKFAYT
ratTMABA-DH  (94)   METINNGK-SIFEARLDVDTSWQCLEYYAGLAASMAGEHIQLPGGSFGYT
 Consensus  (101)   IE I NGK         V    N L YYAG A    G  I L A FAYT
                        151                                               200
NcTMABA-DH  (151)   VKEPLGVCGQIIPWNYPLDMAAWKLGPALCCGNTVVLKLAEQTPLSVLYL
ratTMABA-DH  (143)   RREPLGVCLGIGAWNYPFQIACWKSAPALACGNAMIFKPSPFTPVSALLL
 Consensus  (151)    KEPLGVC I   WNYP  IA WK APAL CGN MI K A  TPLS L L
                        201                                               250
NcTMABA-DH  (201)   AKLIKEAGFPPGVINIINGHGREAGAALVQHPQVDKIAFTGSTTTGKEIM
ratTMABA-DH  (193)   AELYTKAGAPNGLFNVVQG-GAATGQFLCQHRDVAKVSFTGSVPTGMKIM
 Consensus  (201)   A I   AG P GL NIING G   G  L QH  V KIAFTGS  TG  IM
                        251                                               300
NcTMABA-DH  (251)   KMASYTMKNITLETGGKSPLIVFEDADLELAATWSHIGIMSNQGQICTAT
ratTMABA-DH  (242)   EMAAKGTKPITLELGGKSPLIIFSDCNMKNAVKGALLANFLTQGQVCCNG
 Consensus  (251)    MAA  IK ITLE GGKSPLIIF D  L   A IA    QGQIC
                        301                                               350
NcTMABA-DH  (301)   SRILVHEKIYDEFVEKFKAKVQEVSVLGDPFEESTFHGPQVTKAQYERVL
ratTMABA-DH  (292)   TRVFVQKEIADAFTKEVVRQTQRIKIG-DPLLEDTRMGPLINAPHLERVL
 Consensus  (301)   SRI V   I D F      Q I I  DP  E T  GP I      ERVL
                        351                                               400
NcTMABA-DH  (351)   GYINVGKEEGATVMMGGE----PAPQNGKGFFVAPTVFTNVKPTMKIFRE
ratTMABA-DH  (341)   GFVRSAKEQGATVLCGGEPYAPEDPKLKHGYYMTPCILTNCTDDMTCVKE
 Consensus  (351)   GFI  AKE GATVL GGE     P    GFFM P I TN     M   KE
                        401                                               450
NcTMABA-DH  (397)   EIFGPCVAITTFKTEEEALTLANDSMYGLGAALFTKDLTRAHRVAREIEA
ratTMABA-DH  (391)   EIFGPVMSILTFETEAEVLERANDTTFGLAAGVFTRDIQRAHRVAAELQA
 Consensus  (401)   EIFGP MAI TF TE E L  ANDS FGLAAALFTKDI RAHRVA EI A
                        451                                               500
NcTMABA-DH  (447)   GMVWVNSSNDSDFRIPFGGVKQSGIGRELGEAGLAPYCNVKSIHVNLAA-
ratTMABA-DH  (441)   GTCYINNYNVSPVELPFGGYKKSGFGRENGRVTIEYYSQLKTVCVEMGDV
 Consensus  (451)   G  WIN N S   IPFGG K SG GRE G   I  Y NLKSI V LA
                        501
NcTMABA-DH  (496)   -------
ratTMABA-DH  (491)   ESPFENQ
 Consensus  (501)
```

Figure 6.4. Alignment of the cloned *N. crassa* trimethylaminobutyraldehyde dehydrogenase (NcTMABA-DH) to the previously identified rat gene ALDH9.

6.3.5 CLONING AND CHARACTERISATION OF THE *N. crassa* γ-BUTYROBETAÏNE HYDROXYLASE (NcBBH)

Genes coding for γ-butyrobetaïne hydroxylase have been cloned from human (Vaz *et al*, 1998). The sequences of these genes were used to BLAST the *Neurospora* genome database for homologous sequences and led to the identification of one locus

(NCU06891; EMBL accession No. Q7S3G2) with significant homology to that of the human and rat enzymes. The predicted sequence contained 5 exons and codes for an

```
                    1                                                50
humanBBHp    (1)   --------------------------------------------------
   NcBBHp    (1)   MKVDKEAGKETDKIGVNKSDKKAGRKANEETDKLAEAQREFDIQLSRLRN
  ratBBHp    (1)   --------------------------------------------------
Consensus    (1)
                    51                                              100
humanBBHp    (1)   --------------------------------------------------
   NcBBHp   (51)   DLAQLKKSNNKLRKDKGALRLDIVNMKKAFNGVPPTAAVQRDGQLLELYK
  ratBBHp    (1)   --------------------------------------------------
Consensus   (51)
                    101                                             150
humanBBHp    (1)   ---------MACTIQKAEALDGAHLMQILWYDEE----ESLYPAVWLRDN
   NcBBHp  (101)   KIAKEAREFKAGTPQSVEVVDGQQQLVITFAQPDGTTKQVAMSLHWLRDT
  ratBBHp    (1)   ---------MHCAILKAEAVDGARLMQIFWHDGA---ESLYPAVWLRDN
Consensus  (101)            MACTIQKAEAVDGA LMQI WHD D    ESLYPAVWLRDN
                    151                                             200
humanBBHp   (38)   CPCSDCYLDSAKARKLLVEALDVNIGIKGLIFDRKK----------VYIT
   NcBBHp  (151)   CKCPHCVNPDSGQKNFSSTSLPETLEVQSAEVNAADGSVTIVWANDTVST
  ratBBHp   (38)   CQCSDCYLHSAKARKLLLEALDVNIRMDDLTFDQKK----------VYIT
Consensus  (151)   C CSDCYL SAKARKLLLEALDVNI I  L FD KK          VYIT
                    201                                             250
humanBBHp   (78)   WPDEHYSEFQADWLKKRCFSKQARAKLQRELFFPECQYWGSELQLPTLD-
   NcBBHp  (201)   NATSEATTHTSTYDASDIFTWQLPYDLAGNLLPVERTLWDRSKLQAHIDS
  ratBBHp   (78)   WPNGHYSEFEANWLKKRCFSQEARAGLQGELFLPECQYWGSELQLPTLN-
Consensus  (201)   WP  HYSEF A WLKKRCFS QARA LQGELF PECQYWGSELQLPTLD
                    251                                             300
humanBBHp  (127)   ------FEDVLRYDEHAYKWLSTLKKVGIVRLTGASDK----PGEVSKLG
   NcBBHp  (251)   GDLRVSYNDWLTSDAAFWKAFESLARFGILFVHSIPSDRALVESQVEKIA
  ratBBHp  (127)   ------FEDVLNDDDHAYKWLSSLKKVGIVRLTGAADK---RGEIIKLG
Consensus  (251)         FEDVL  DDHAYKWLSSLKKVGIVRLTGAADK     GEV KLG
                    301                                             350
humanBBHp  (167)   KRMGFLYLTFYGHTWQVQDKIDANNVAYTTGKLSFHTDYPALHHPPGVQL
   NcBBHp  (301)   NRIGILMHTFYGHTWDVRSKPRAENVAYTNVFLGLHQDLMYIDPPPRLQL
  ratBBHp  (167)   KRIGFLYLTFYGHTWQVQDKIDANNVAYTTGKLSFHTDYPALHHPPGVQL
Consensus  (301)   KRIGFLYLTFYGHTWQVQDKIDANNVAYTTGKLSFHTDYPALHHPPGVQL
                    351                                             400
humanBBHp  (217)   LHCIKQTVTGGDSEIVDGFNVCQKLKKNNPQAFQILS---STFVDFTDIG
   NcBBHp  (351)   LHCISNSFQGGESLFSDGARAAYSLELNNPLAFDQLRGNRSPQFHYHRNG
  ratBBHp  (217)   LHCIKQTVTGGDSEIVDGFNVCQKLKEKNPQAFSILS---STFVDFTDIG
Consensus  (351)   LHCIKQTVTGGDSEIVDGFNVCQKLK NNPQAF ILS    STFVDFTDIG
                    401                                             450
humanBBHp  (264)   VDYCDFSVQSKHKIIELDDKGQVVRINFN--------------------
   NcBBHp  (401)   NDYHMGRNTFRYAGRTGEGKGFLSRIHWAPPFQAPFSRQTGATATNVLGN
  ratBBHp  (264)   VDYCDFSVQSKHKIIELDDKGQVVRINFN--------------------
Consensus  (401)   VDYCDFSVQSKHKIIELDDKGQVVRINFN
                    451                                             500
humanBBHp  (293)   -------------NATRDTIFDVPVERVQPFYAALKEFVDLMNSKESKFTF
   NcBBHp  (451)   RVIQDGGGGAYAENEHAVVEEKGKNMTKWVPAAKEFERELSAEENMFEL
  ratBBHp  (293)   -------------NATRDTVFDVPIERVQPFYAALKEFVDLMNSKEYKYTF
Consensus  (451)               NATRDTVFDVPIERVQPFYAALKEFVDLMNSKE KFTF
                    501                                             550
humanBBHp  (331)   KMNPGDVITFDNWRLLHGRR---SYEAGTEISRHLEGAYADWDVVMSRLR
   NcBBHp  (501)   KMKEGECVIFDNWRVLHGRREFQTEGQAEGAERWLKGTYISHQVYKAMED
  ratBBHp  (331)   KMNPGDVITFDNWRLLHGRR---SYEAGTEISRHLEGAYADWDVVMSRLR
Consensus  (501)   KMNPGDVITFDNWRLLHGRR   SYEAGTEISRHLEGAYADWDVVMSRLR
                    551                                             600
humanBBHp  (378)   ILRQRVENGN----------------------------------------
   NcBBHp  (551)   KLQWKLAQKEGGIPLAIGVAEAHGLGWKERGQLPKKEAPKQETTAPVQPK
  ratBBHp  (378)   ILRQRVMNGN----------------------------------------
Consensus  (551)   ILRQRV NGN
                    601                                             650
humanBBHp  (388)   --------------------------------------------------
   NcBBHp  (601)   EEAPKVEEAPKVEETPKVEEAPKVEEAPKVEEAPKVEEAPKVEEAVKPEE
  ratBBHp  (388)   --------------------------------------------------
Consensus  (601)
                    651               671
humanBBHp  (388)   --------------------
   NcBBHp  (651)   AQNEGPSRQPKEQLGTVNWNA
  ratBBHp  (388)   --------------------
Consensus  (651)
```

Figure 6.5. Alignment of the cloned fragment of the *N. crassa* γ-butyrobetaïne hydroxylase (NcBBH; NCU06891) to the similar genes cloned from human and rat.

mRNA product of 3786 bp, with the resulting protein predicted to contain 1262 amino acids. This is considerably larger than the rat and human copies which both consist of 387 amino acids. Furthermore, only the last exon of the predicted ORF is homologous to the mammalian enzymes. Several attempts were made to amplify the full predicted fragment from *N. crassa* cDNA; however, the only primer set that could be used successfully amplified the region encoded by the last exon. The size of the amplified product was 2016 bp, which would lead to a predicted protein of 672 amino acids and a molecular weight of 75.2 kDa. Regions from the central part of the predicted amino acid sequence from the *N. crassa* BBH (excluding 111 aa on the N-terminal and 110 aa on the C-terminal) share similarity with the human and rat genes (Figure 6.5.). The C-terminal domain contains a six-fold repeat of the sequence "PKVEE".

6.3.6 CARNITINE PRODUCTION BY TRANSGENIC *S. cerevisiae* STRAINS

To asses the ability of the generated strains to produce carnitine, strains were inoculated onto plates containing ethanol as carbon source (SCE) and covered with a mat of FY23Δ*cit2* cells. Since the Δ*cit2* strain cannot grow on non-fermentable carbon sources, such as ethanol, without exogenous supplementation of carnitine, the production of carnitine by a nearby colony allows growth to occur leading to the formation of a halo of growing Δ*cit2* cells around the colony. This provides for a straightforward means of screening for strains able to produce carnitine. Similar amounts of the engineered yeast strains containing the various carnitine biosynthesis genes were inoculated on the described plates, and also plates supplemented with the precursors γ-butyrobetaïne (20 mgL^{-1}), trimethyllysine (20 mgL^{-1}) and also with lysine and methionine (added to final concentrations of 200 and 500 mg L^{-1} of each amino acid). The plates were incubated at 30 °C for 8 days and monitored for halo formation. As can be expected, no growth of Δ*cit2* cells was visible on the unsupplemented SCE plate which served as a control (Figure 6.6.). Supplementation with γ-butyrobetaïne resulted in a halo of Δ*cit2* growth around all strains which contained the integrated NcBBH gene, confirming the functional expression thereof in yeast. SCE plates supplemented with trimethyllysine resulted in halo formation in the yeast strain which contain all four carnitine biosynthesis genes, FY23 TMLH HTMLA TMABA-DH BBH and also FY23 CB, which contains all four genes and also the *N. crassa* free lysine methyltransferase. Interestingly, a yeast strain expressing only BBH, TMLH and

HTMLA, excluding TMABA-DH also produced carnitine resulting in visible growth of Δ*cit2*. Supplementation with lysine and methionine, in order to assess the functionality of the cloned free lysine methyltransferase did not lead to halo formation.
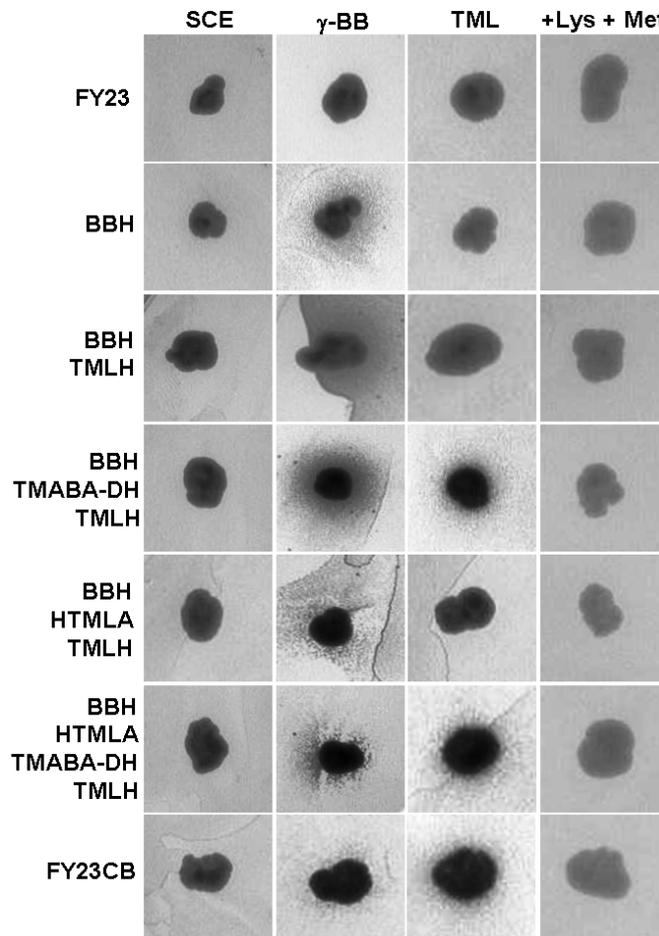
Figure 6.6. Engineered *S. cerevisiae* strains producing carnitine lead to the formation of a halo of growth around the spotted colony, as a result of the complementation of the carnitine dependent Δ*cit2* strain. SCE plates were supplemented with either γ-butyrobetaïne (γ-BB; 20 mgL^{-1}), trimethyllysine (TML; 20 mgL^{-1}) or lysine and methionine (500 mgL^{-1}). Unsupplemented (SCE) plates were used as control.

6.4. DISCUSSION

In the fungus *N. crassa*, as in higher eukaryotes, carnitine is synthesized via four sequential enzymatic conversions of the precursor trimethyllysine. The precursor trimethyllysine either originates from the release of trimethylated lysine residues liberated after protein degradation or is produced, as is uniquely the case in *N. crassa*, by the sequential enzymatic methylation of free lysine. This conversion of lysine to trimethyllysine is catalyzed by the *N. crassa* free lysine methyltransferase (FLMT).

The four central carnitine biosynthesis genes were successfully cloned from cDNA extracted from growing cultures of *N. crassa* and heterologously expressed in the

laboratory yeast strain FY23. The generated strains were assessed for carnitine production in a plate based screen. This analysis allows only for the preliminary detection of carnitine production and does not provide quantitative insight into levels of carnitine produced. The data indicate that the gene encoding a γ-butyrobetaïne hydroxylase is functionally expressed in *S. cerevisiae* and is able to convert γ-butyrobetaïne to L-carnitine in all transformed strains. The sequential integration of the remaining three biosynthesis genes as well as of the free lysine methyl transferase did not have negative impact on BBH functionality. Furthermore, a strain expressing all four genes from the *N. crassa* carnitine biosynthesis was able to convert supplemented trimethyllysine to L-carnitine. This is the first report of a genetically engineered strain of *S. cerevisiae* that is able to neo-synthesize L-carnitine. It should be noted that in the assay system that was used, no carnitine production was observed without trimehtyllysine supplementation in a strain bearing all four biosynthesis genes. This indicates that the physiological concentration of trimethyllysine in yeast is likely to be insufficient for the effective production of carnitine by the carnitine biosynthesis pathway. Interestingly, the presence of the gene encoding TMABA-DH was not required for this conversion to take place. This could indicate that one of the native yeast aldehyde dehydrogenases is able to perform the conversion from trimethylaminobuteraldehyde to γ-butyrobetaïne. This would be a reasonable explanation, since the native *S. cerevisiae* aldehyde dehydrogenases do share significant similarity with the gene cloned from *N. crassa*. From the plate assays used in this study, it is clear that the amount of trimethyllysine that is naturally derived from protein degradation in *S. cerevisiae* is insufficient to lead to halo formation, since this phenomenon is only observed when TML is added to the media. The addition of the gene encoding the *N. crassa* free lysine methyltransferase did not result in halo formation, even when lysine and methionine were added to the medium. While this observation certainly suggests that this first step in the pathway is limiting carnitine production in this strain, the lack of sensitivity of this preliminary assay does not allow drawing further conclusions regarding the functionality of the expressed gene. It is possible that there is insufficient S-adenosyl methionine to perform the sequential methylations of the supplemented lysine. However, *S. cerevisiae* is known to accumulate large quantities of SAM upon methionine supplementation (Gawel *et al*, 1962). A second possibility is that L-carnitine is produced at amounts that are not sufficient to ensure growth of the plated matt of Δ*cit2* cells. Clearly, a more quantitative

and sensitive measurement of carnitine production is required. Current efforts are focused on standardizing a LC-MS based analytical technique for the measurement of L-carnitine and all the intermediates of the carnitine biosynthesis pathway.

6.5. REFERENCES

Borum, P. R. and Broquist, H. P. (1977). Purification of S-adenosylmethionine: epsilon-N-L-lysine methyltransferase. The first enzyme in carnitine biosynthesis. *J Biol Chem* **252,** 5651-5.

Calabrese, V., Giuffrida Stella, A. M., Calvani, M. and Butterfield, D. A. (2006). Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes. *J Nutr Biochem* **17,** 73-88.

CASEY, G. P., W. XIAO and G. H. RANK. (1988). A convenient dominant selection marker for gene transfer in industrial yeast strains of *Saccharomyces cerevisiae*: *SMR1* encoded resistance to the herbicide sulfometron methyl. *J Inst Brew* **94,** 93-97.

Chern, M. K. and Pietruszko, R. (1995). Human aldehyde dehydrogenase E3 isozyme is a betaine aldehyde dehydrogenase. *Biochem Biophys Res Commun* **213,** 561-8.

Davis, R.H. (2000). *Neurospora*: Contributions of a model organism. Oxford University Press, Oxford, UK.

Davis, R.L., deSerres, D. (1970). Genetic and microbial research techniques for *Neurospora crassa*. *Method Enzymol* **27A,** 79-143.

Dunn, W. A., Rettura, G., Seifter, E. and England, S. (1984). Carnitine biosynthesis from gamma-butyrobetaine and from exogenous protein-bound 6-N-trimethyl-L-lysine by the perfused guinea pig liver. Effect of ascorbate deficiency on the in situ activity of gamma-butyrobetaine hydroxylase. *J Biol Chem* **259,** 10764-70.

Gawel, L. J., Turner, J. R. and Parks, L. W. (1962). Accumulation of S-adenosylmethionine by microorganisms. *J Bacteriol* **83,** 497-9.

Henderson, L. M., Nelson, P. J. and Henderson, L. (1982). Mammalian enzymes of trimethyllysine conversion to trimethylaminobutyrate. *Fed Proc* **41,** 2843-7.

Kikonyogo, A. and Pietruszko, R. (1996). Aldehyde dehydrogenase from adult human brain that dehydrogenates gamma-aminobutyraldehyde: purification, characterization, cloning and distribution. *Biochem J* **316 (Pt 1),** 317-24.

Kurys, G., Shah, P. C., Kikonygo, A., Reed, D., Ambroziak, W. and Pietruszko, R. (1993). Human aldehyde dehydrogenase. cDNA cloning and primary structure of the enzyme that catalyzes dehydrogenation of 4-aminobutyraldehyde. *Eur J Biochem* **218,** 311-20.

LaBadie, J., Dunn, W. A. and Aronson, N. N., Jr. (1976). Hepatic synthesis of carnitine from protein-bound trimethyl-lysine. Lysosomal digestion of methyl-lysine-labelled asialo-fetuin. *Biochem J* **160,** 85-95.

Lin, S. W., Chen, J. C., Hsu, L. C., Hsieh, C. L. and Yoshida, A. (1996). Human gamma-aminobutyraldehyde dehydrogenase (ALDH9): cDNA sequence, genomic organization, polymorphism, chromosomal localization, and tissue expression. *Genomics* **34,** 376-80.

Longo, N., Amat di San Filippo, C. and Pasquali, M. (2006). Disorders of carnitine transport and the carnitine cycle. *Am J Med Genet C Semin Med Genet* **142C,** 77-85.

Moses, S. B., Otero, R. R. and Pretorius, I. S. (2005). Domain engineering of *Saccharomyces cerevisiae* exoglucanases. *Biotechnol Lett* **27,** 355-62.

Ramsay, R. R., Gandour, R. D. and van der Leij, F. R. (2001). Molecular enzymology of carnitine transfer and transport. *Biochim Biophys Acta* **1546,** 21-43.

Siebert, P. D. and Chenchik, A. (1993). Modified acid guanidinium thiocyanate-phenol-chloroform RNA extraction method which greatly reduces DNA contamination. *Nucleic Acids Res* **21,** 2019-20.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122,** 19-27.

Stein, R. and Englard, S. (1981). The use of a tritium release assay to measure 6-N-trimethyl-L-lysine hydroxylase activity: synthesis of 6-N-[3-3H]trimethyl-DL-lysine. *Anal Biochem* **116,** 230-6.

Strijbis, K., Van Roermund, C.W.T., Hardy, G.P., Van den Berg, J., Bloem, K., De Haan, J., Van Vlies, N., Wanders, R.J.A., Vaz, F.M., Distel, B. (2009) Identification and characterisation of a complete carnitine biosynthesis pathway in *Candida albicans*. *FASEB J* **23,** 1-11.

Swiegers, J. H., Dippenaar, N., Pretorius, I. S. and Bauer, F. F. (2001). Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: three carnitine acetyltransferases are essential in a carnitine-dependent strain. *Yeast* **18,** 585-95.

Swiegers, J. H., Vaz, F. M., Pretorius, I. S., Wanders, R. J. and Bauer, F. F. (2002). Carnitine biosynthesis in *Neurospora crassa*: identification of a cDNA coding for epsilon-N-trimethyllysine hydroxylase and its functional expression in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **210,** 19-23.

Tein, I., Bukovac, S. W. and Xie, Z. W. (1996). Characterization of the human plasmalemmal carnitine transporter in cultured skin fibroblasts. *Arch Biochem Biophys* **329,** 145-55.

Van Roermund C.W., Elgersma, Y., Singh, N., Wanders, R.J., Tabak, H.F. (1995) The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. *EMBO* **14,** 3480-3486

Vaz, F. M., Fouchier, S. W., Ofman, R., Sommer, M. and Wanders, R. J. (2000). Molecular and biochemical characterization of rat gamma-trimethylaminobutyraldehyde dehydrogenase and evidence for the involvement of human aldehyde dehydrogenase 9 in carnitine biosynthesis. *J Biol Chem* **275,** 7390-4.

Vaz, F. M., Ofman, R., Westinga, K., Back, J. W. and Wanders, R. J. (2001). Molecular and Biochemical Characterization of Rat epsilon -N-Trimethyllysine Hydroxylase, the First Enzyme of Carnitine Biosynthesis. *J Biol Chem* **276,** 33512-7.

Vaz, F. M., van Gool, S., Ofman, R., Ijlst, L. and Wanders, R. J. (1998). Carnitine biosynthesis: identification of the cDNA encoding human gamma-butyrobetaine hydroxylase. *Biochem Biophys Res Commun* **250,** 506-10.

Vaz, F. M. and Wanders, R. J. (2002). Carnitine biosynthesis in mammals. *Biochem J* **361,** 417-29.

Chapter 7

General discussion and future perspectives.

7.1. CONCLUDING REMARKS

The role of carnitine and of the carnitine shuttle in the transfer of activated acyl groups between intracellular compartments has been clearly defined. In the yeast, *Saccharomyces cerevisiae*, the enzymes involved have previously been isolated in genetic screens using the carnitine dependen phenotype of a Δ*cit2* strain, which is relies on the function of the carnitine shuttle to sustain growth on non-fermentable carbon sources (such as ethanol, acetate and glycerol) and fatty acids. From studies in mammalian systems its has, however, become increasingly clear that the modulatory effect of the shuttle has a far reaching impact on the regulation of metabolic homeostasis and that shuttle independent activities of carnitine do exist (reviewed in Chapter 2). With this as background, the major aim of this study was to shed light on the fundamental question of what the function of carnitine and also the three CATs are within the framework of a eukaryotic cell.

In yeast, three separate carnitine acetyl-transferases (CATs) have been identified and shown to be required for the integrity of the carnitine shuttle (Swiegers *et al*, 2001). The work done in this study indicates that each of these enzymes are located in separate cellular compartments and have specific functions within the shuttle and that, at least for Cat2p, shuttle independent functions do exist. Although the exact roles of these enzymes in the context of the shuttle and yeast metabolism in general remains to be defined, this does give a clear indication of the tight regulation that is required to maintain the function of the carnitine shuttle.

Considering that yeast is able to metabolize non-fermentable carbon sources in the cytosol to generate acetyl-CoA, the presence of cytoslic CAT activity would be required to enable the trafficking of acetyl groups to the mitochondria. This does not, however, explain the requirement of two CATs (Yat1p and Yat2p) that would be able to catalyze the cytosolic reaction. In addition, enzymatic carnitine-acetyltransferase assays previously done in this laboratory, using both a spectrophotometric method and also an ES-MS based method was unable to detect CAT activity for either Yat1p or Yat2p and indicated Cat2p to be responsible for at least 95 % of total CAT activity in cultures grown on all non-fermentable carbon sources and fatty acids (data not shown). These assays did however not address the possible effect of carnitine on the regulation of CAT activity. In fact, there is no data, transcriptional or post-translational, available on regulation of these enzymes in general and future studies on these aspects will greatly

add to the understanding of their specific functions. Furthermore, data describing the effect of the three yeast CATs on the regulation of carbon metabolism is required to gain a holistic understanding of the roles of the enzymes and the influence of the carnitine shuttle in general.

Recently, an interesting report from studies on the *Candida albicans* CATs indicated that complementation of the *S. cerevisiae* CAT mutants results in intriguing differences and similarities between the two species. *CTN2* (homologous to *CAT2*) was found to not to be functional in *S. cerevisiae*, whereas, *CTN3* (homologous to *YAT2*) was able to complement deletion of *YAT2* in combination with *CIT2*. On the other hand, *CTN1* (homologous to *YAT1*) was able to restore growth in both a Δ*cit2*Δ*yat1* and also a Δ*cit2*Δ*yat2* strain (Zhou and Lorenz, 2008). It would be of interest to investigate the role of specific regions/domains of the *C. albicans CTN1* protein in the complementation of both *YAT1* and *YAT2*. This could shed light on the specific and also distinct roles of Yat1p and Yat2p. Future studies on the *S. cerevisiae* CATs would also be greatly aided by the identification of interacting protein partners. This would advance the understanding of the enzymes function within the context of the carnitine shuttle and also the shuttle independent functions, such as described for *CAT2* with regards to oxidative stress.

The work done in Chapter 3 also led to the initial description of a role for carnitine, independent of the carnitine shuttle, in the protection against oxidative cellular stresses (Franken *et al*, 2008). Although, several clinical studies have based conclusions on the assumption that carnitine is an antioxidant, the results of this study indicates that even though carnitine behaves similar to known antioxidants within a biological context, the molecule itself does not possess free radical scavenging activity (Chapter 4). Furthermore, the data also indicate carnitine to have toxic effects in combination with thiol modifying agents. This is the first report of a detrimental outcome associated with carnitine supplementation. These findings should certainly be taken into account when considering potential therapeutic applications.

It is interesting to note that supplementation of carnitine protects growing cells against H_2O_2 insult, but does not protect against a sudden oxidative shock, induced under non-growing conditions. This signifies that the effect of carnitine present in the media is not due to an enhanced state of cellular protection, such as reported from studies in neuronal systems, but rather a consequence of either stimulating growth or delaying cell death (Calabrese *et al*, 2006). It seems unlikely that carnitine would be

able to enhance growth of yeast cultures, since no such an effect has been observed in any media condition that has been tested to date. Furthermore, the dual function (protective and detrimental) of carnitine is reminiscent of the divergent outcomes observed for carnitine supplementation in apoptosis, when comparing healthy to cancerous cells (Wenzel *et al*, 2005; Ferrara *et al*, 2005). In addition, the function of cytochrome c is required for both phenotypic outcomes. These observations in combination with global gene expression analysis of carnitine treated cultures indicate a possible role for carnitine in delaying the onset of programmed cell death. Future work should establish whether the phenotype of apoptosis is affected by carnitine supplementation. In addition, it would be of importance to investigate processes, such as ageing, that are affected by the build-up of reactive oxygen species.

The most intriguing question that arises from this work is how a molecule that functions in a specific and tightly regulated metabolic niche would have specific and evolutionary conserved impacts on cellular stresses. It would be of great interest to identify the upstream regulators of the effect of carnitine under these conditions. Promising targets identified from the microarray analysis (Chapter 5) are the conserved kinases Tor1p and Sch9p and also regulators of iron homeostasis, such as Aft2p. Furthermore, this also raises the question of what generates the signal that results in these specific outcomes. Since the protective effect of carnitine is independent of shuttle activity, carnitine itself does appear to be directly involved as an effector. It would be interesting to screen the yeast proteome, using bioinformatics based methods, for proteins containing sites resembling the carnitine binding site present in CATs. A ligand-binding protein array study could also be considered with the same goal in mind.

The second part of the work presented in this thesis formed part of an applied study that focused on the creation of yeast strains that are able to neo-synthesize L-carnitine, by heterologously expressing the four genes involved in carnitine biosynthesis from *Neurospora crassa* (Chapter 6). The data indicates that carnitine production is indeed achieved by the engineered strains expressing the genes involved in this pathway. Quantitative data on the amounts of carnitine and the intermediates of the pathway will answer questions regarding the functionality of the *N. crassa* free lysine methyltransferase and also give insight to the metabolic flux through the engineered system. This should allow the implementation of strategies aimed at maximizing carnitine production. The eventual aim of this project envisions the establishment of carnitine producing industrial yeast strains that could be used in a variety of potential

commercial applications, such as fermented products and single cell proteins with enhanced carnitine content. The main obstacle in this project will likely be to obtain sufficient amounts of synthesized carnitine in order to have a benefit in specific products. An additional consideration that stems out of the work presented is the possibility that carnitine producing strains could be more resistant to the stresses encountered in industrial applications, such as wine making. This would indeed be an avenue worthwhile exploring.

7.2 REFERENCES

Calabrese, V., Giuffrida Stella, A. M., Calvani, M. and Butterfield, D. A. (2006). Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes. *J Nutr Biochem* **17,** 73-88.

Ferrara, F., Bertelli, A. and Falchi, M. (2005). Evaluation of carnitine, acetylcarnitine and isovalerylcarnitine on immune function and apoptosis. *Drugs Exp Clin Res* **31,** 109-14.

Franken, J., Kroppenstedt, S., Swiegers, J. H. and Bauer, F. F. (2008). Carnitine and carnitine acetyltransferases in the yeast *Saccharomyces cerevisiae*: a role for carnitine in stress protection. *Curr Genet* **53,** 347-60.

Swiegers, J. H., Dippenaar, N., Pretorius, I. S. and Bauer, F. F. (2001). Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: three carnitine acetyltransferases are essential in a carnitine-dependent strain. *Yeast* **18,** 585-95.

Wenzel, U., Nickel, A. and Daniel, H. (2005). Increased carnitine-dependent fatty acid uptake into mitochondria of human colon cancer cells induces apoptosis. *J Nutr* **135,** 1510-4.

Zhou, H. and Lorenz, M. C. (2008). Carnitine acetyltransferases are required for growth on non-fermentable carbon sources but not for pathogenesis in *Candida albicans*. *Microbiology* **154,** 500-9.