A new role for carnitine in yeasts

by

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DECLARATION

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

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SUMMARY

L-Carnitine (3-0H-4-N-trimethylaminobutanoic acid), also called vitamin B_T, is required for the metabolism of fatty acids. Only one specific metabolic activity has been ascribed to L-carnitine in eukaryotic organisms, the transfer of activated acyl residues. In the case of yeast, this process involves the transfer of activated acetyl residues from the peroxisomes or the cytoplasm to the mitochondria. In *Saccharomyces cerevisiae*, β-oxidation of fatty acids takes place exclusively in the peroxisomes. The process generates peroxisomal acetyl-CoA, and the activated acetyl-residue has to be transferred to the mitochondria for energy production. Acetyl-CoA and other acyl-CoAs however can not be transferred across intracellular membranes. The activated acetyl residue is therefore transferred to a molecule of carnitine to form acetyl carnitine, which can be shuttled across membranes. The reverse reaction, the transfer of the activated acetyl to free CoA-SH and the liberation of carnitine takes place in the mitochondria. This process is also referred to as the carnitine shuttle.

Most organisms, including some yeast, fungi, plants and all mammals, but not *S. cerevisiae*, can synthesize carnitine from lysine and S-adenosyl-methionine. However, in humans, carnitine synthesis is insufficient to satisfy carnitine requirements, and dietary contributions are essential. Various diseases linked to carnitine deficiencies have been described. Such deficiencies include those found in neonates who, in the absence of carnitine, are unable to assimilate fatty acids from milk, or genetically inborn errors of metabolism, frequently linked to a defective transport of carnitine into cells.

More recent literature suggests that carnitine supplementation can have beneficial effects in a number of pathologies, and can also provide some protection against diabetes and liver disease. It has furthermore been suggested that carnitine can contribute to slowing brain aging and to improve conditions of patients suffering from neurodegenerative diseases such as Alzheimer's disease. The accumulation of such data may suggest that carnitine plays additional, as yet unrecognized roles in cellular physiology.

In the study reported here, the yeast *S. cerevisiae* was used to identify possible additional roles for carnitine in cellular metabolism. The study furthermore attempted to identify genes that may be associated with such additional roles. The data show that carnitine supplementation of the growth substrate can protect yeast cells from hyper osmotic and high temperature stress. These protective effects are independent of the metabolic role of carnitine, since deletion of genes that are essential for the carnitine shuttle does not reduce the protective effect. The investigation also suggests that there are no other metabolic roles for carnitine in yeast than the carnitine shuttle, and that it therefore may act as a compatible solute in osmo-protection. The data also indicate a role for *PHO87*, previously identified as a low affinity inorganic phosphate carrier, in the protective effect of carnitine. *PHO87* overexpression strains accumulate higher concentrations of carnitine, whereas *pho87*Δ strains contain less carnitine than the corresponding wild type strain. The data therefore suggest either a direct or a regulatory role of the protein in carnitine uptake.

OPSOMMING

L-Karnitien (3-0H-4-N-trimetielaminobutaansuur), ook bekend as vitamien B_T, word vir die metabolisme van vetsure benodig. Tot dusver is net een spesifieke metaboliese aktiwiteit in eukariotiese organismes aan L-karnitien toegeskryf, naamlik die oordrag van geaktiveerde asielreste. In die geval van gis behels hierdie proses die oordrag van geaktiveerde asetielreste vanaf die peroksisome of die sitoplasma na die mitochondria. In *Saccharomyces cerevisiae* vind die β-oksidasie van vetsure uitsluitlik in die peroksisome plaas. Hierdie proses genereer peroksisomale asetiel-KoA, en die geaktiveerde asetielreste moet vir energieproduksie na die mitochondria oorgeplaas word. Asetiel-KoA en ander asiel-KoA's kan egter nie oor intrasellulêre membrane vervoer word nie. Die geaktiveerde asetielreste word dus na 'n karnitienmolekuul oorgedra om asetielkarnitien te vorm, wat oor die membrane vervoer kan word. Die omgekeerde reaksie, naamlik die oordrag van die geaktiveerde asetiel na vrye KoA-SH en die vrystelling van karnitien, vind in die mitochondria plaas. Hierdie proses staan ook as die "karnitien heen-en-weerstelsel" bekend.

Die meerderheid organismes, insluitende sommige giste, swamme, plante en alle soogdiere, hoewel nie *S. cerevisiae* nie, kan karnitien vanaf lisien en S-adenosiel-metionien sintetiseer. In mense is karnitiensintese egter onvoldoende om in die karnitienbehoeftes te voorsien en voedingsaanvullings is dus noodsaaklik. Verskeie siektes wat met 'n tekort aan karnitien verband hou, is reeds beskryf. Sulke tekorte kom o.a. voor in pasgebore babas wat in die afwesigheid van karnitien nie die vetsure afkomstig van melk kan opneem nie, of in persone met genetiese afwykings in hul metabolisme wat in baie gevalle met die onvoldoende vervoer van karnitien na die selle verband hou.

In onlangse literatuur word daar voorgestel dat karnitienaanvulling voordelig vir 'n aantal patologiese toestande kan wees, en dat dit ook 'n mate van beskerming teen diabetes en lewersiektes kan verskaf. Daar is ook beweer dat karnitien die veroudering van die brein kan teëwerk en die toestand van pasiënte wat aan neurodegeneratiewe siektes soos Alzheimer's ly, kan verbeter. Hierdie data dui dus op die moontlikheid dat karnitien bykomende, en tot dusver onbekende, funksies in sellulêre fisiologie kan hê.

In die studie waaroor hier verslag gedoen word, is die gis *S. cerevisiae* gebruik om moontlike bykomende funksies vir karnitien in sellulêre metabolisme te identifiseer. Die studie poog om gene wat moontlik met sulke addisionele funksies geassosieer kan word, te identifiseer. Die data toon dat karnitienaanvulling van die groeisubstraat die gisselle teen hiperosmotiese en hoë-temperatuur stres kan beskerm. Hierdie beskermende invloed is onafhanklik van die metaboliese funksie van karnitien, aangesien die delesie van die gene wat noodsaaklik is vir die "karnitien heen-en-weerstelsel" nie die beskermende effek verminder nie. Hierdie ondersoek stel ook voor dat, buiten karnitientransport, daar geen ander metaboliese rolle vir karnitien in gis is nie, en dat dit dus moontlik as 'n verenigbare opgeloste stof in osmo-beskerming kan optree. Die data dui ook op 'n funksie vir *PHO87*, wat voorheen as 'n lae-affiniteit anorganiese fosfaatdraer in die beskermende effek van

karnitien geïdentifiseer is. *PHO87*-ooruitdrukkingsrasse akkumuleer hoër karnitienkonsentrasies, terwyl *pho87*Δ-rasse minder karnitien as die ooreenkomstige wilde ras bevat. Die data dui dus op óf 'n direkte óf 'n regulatoriese funksie vir die proteïen in karnitienopname.

BIOGRAPHICAL SKETCH

Candide Font-Sala was born on 5 December 1973 in France. She passed the Maitrise-degree that is equivalent to the South African HonsBSc-degree. Under the supervision of Prof FF Bauer, she started her MSc in Wine Biotechnology at the University of Stellenbosch during 1999.

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature review**
Carnitine roles and functions

Chapter 3 **Research Results**
Investigating the effects of L-carnitine on yeasts physiology:
A new role for carnitine in yeasts
The determination of carnitine acetyltransferase activity in *Saccharomyces cerevisiae* by HPLC-electrospray mass spectrometry (appendix)

Chapter 4 **General Discussion and Conclusions**

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CHAPTER 1

INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Saccharomyces cerevisiae is one of the most studied of all organisms, and was the first eukaryote whose genome was fully sequenced. As a consequence, this yeast currently is probably the best understood eukaryotic model system with the widest set of molecular tools. Being an eukaryote, the cellular organization is similar to more complex organisms, and many yeast proteins have their mammalian equivalent. The degree of genetic conservation between yeast and humans can allow some extrapolation from data obtained in yeast to generate insights into human genetics (Dujon, 1996). Many human genes can be expressed in *S. cerevisiae* and complement the corresponding yeast gene deletion mutants. Genetically modified yeasts are cultured to produce pharmaceutical molecules at high levels that are used in human medicine as therapeutic products (Table 1.1) and in the production of amino-acids, vitamins, organic acids and other compounds (Huang *et al.*, 1996).

Table 1.1 Emerging biotechnologies exploiting yeasts activity, functions and genes (adapted from Walker, 1998).

Research field	Examples of the value of yeast research
Oncology	• Molecular mechanism of cell cycle determined using budding and fission yeasts. Keys to our understanding of cancer cell division. • Biomedical regulation of certain human oncogenes e.g. *ras* (*S. cerevisiae*). • p53 mutation studies (implicated in tumorgenesis). • Telomere-binding proteins function provided insight on cancer cells maintenance of a constant average telomere length.
Pharmacology and toxicology	• Apoptotic cell studies (*S. cerevisiae*). • Genetic and Biochemistry multidrug resistance. • Studies of drug action (anti-tumor agents). • Drug metabolism, drug-drug interactions and pharmaco-kinetics. • Drugs screening and mutagens/genotoxic agents' tests. • Mycotoxins, xenoestrogens, etc. assays. • Detection of toxic chemicals in pollutant samples using yeast biosensors.
Virology	• *S. cerevisiae* a virus model (killer plasmids and retrotransposons); anti-viral components (surface antigens for vaccines); system for studying viral protein action; two-hybrid system to study self interaction of viral proteins.
Genetics	• Yeast artificial chromosomes (YACs) are invaluable in cloning human DNA and in mapping the human genome.
Neurodegenerative disease	• Research into yeast prions is offering new insights into the molecular biology of protein-based inheritance and possible therapy for certain neurodegenerative human diseases (kuru, Creutzfeld-Jacob, fatal familial insomnia).

In fundamental biological research, yeast has contributed tremendously to our understanding of cellular biology, genetics, biochemistry and molecular biology. In the more applied areas of biomedical research (Table 1.2), *S. cerevisiae* has helped to increase our understanding of diseases ranging from cancer to AIDS, contributed to drug screening and development, as well as to the studies of drug metabolism and genotoxicity screens. Yeast also has proven useful to better understand some human genetic disorder and today is used for the production of pharmaceuticals, vaccines, probiotics, hormones and blood factors (Burden and Eveleigh, 1990; Mattey, 1992; Wainwright, 1992; Stahmann, 1997).

Table 1.2 Non-exhaustive list of cloned therapeutic proteins synthesized by yeasts (adapted from Walker, 1998; Bathrust, 1994; Hadfield *et al.*, 1993; Wiseman, 1996; Rallabhandi and Yu, 1996).

Donor DNA source	Examples of gene products
Prokaryotic	•Tetanus toxin fragment C; streptokinase; whooping cough antigen.
Viral	•Various genes encoding surface antigens, enzymes and other proteins from virus: Hepatitis B; Herpes simplex; HIV; Foot and mouth; Influenza; Polio; Bovine leukemia; Polyoma; Epstein-Barr; Oncogenic retroviruses.
Protozoa	•Malaria antigen.
Animal	•Leech hirudin; viper echistatin; porcine interferon; rabbit α-globin; porcine urokinase; bovine pancreatic typsine inhibitor (aprotinin); rat glia-derivated nexin; bovine and mouse interleukin.
Human	• Hormones: insulin; parathyroid hormone; human chorionic gonadotropin; human growthhormone; somatostatin. • Antibodies: functional antibodies and Fab fragments; chorismate mutase "abzyme" antibody; IgE receptor. Growth factors: insulin-like growth factor (IGF 1); nerve growth factor; epidermal growth facor; tissue factor; platelet-derivated endothelial growth factors; interleukins; macrophage-colony stimulating factor; leukine GM-CSF; tumor necrosis factor. • Interferons: leukocytes interferon-alpha(D); interferon-alpha-2, -beta 1 and hybrid X-430. Blood proteins: haemoglobin; factor VIII and XIII; erythropoietin; serum albumin; antithrombin III; alpha 1-antitrypsin; tissue plasminogen activator; fibrinogen; lactoferrin. • Enzymes/inhibitors: proteinase inhibitor 6; gastric lipase; thyroid peroxidase; pro-urokinase; salivary α-amylase; lysozyme; elastase inhibitor (Elafin); liver epoxide hydrolase; Cu, Zn, superoxide dismutase; cytochrome P450. • Others: oestrogen receptor; cystic fibrosis transmembrane conductance regulator (CFTR); *CDC 28* and G1 cyclin homologues; cancer cell surface antigens; β-endorphin.

Many of these new xenoproteins and molecules of therapeutical interest are being developed for future clinical use. In the future the hope lies in yeast genome research to find easy and practical applications in the diagnosis and therapy of human disorders as

fundamental and applied research on yeast contributes to the understanding of cancer and inborn errors of metabolism (De Simone and Famularo, 1997; Walker, 1998).

Among those inborn errors of metabolism are carnitine deficiencies. Carnitine is a small, water-soluble quaternary ammonium compound, which exists in two enantiomeric forms, L- or D-carnitine. In eukaryotes, only the L-form is synthesized and metabolically active. In this thesis, and unless otherwise indicated, the word carnitine will therefore always refer to L-carnitine.

Carnitine has an important role in energy-generating metabolic processes. Healthy adults adopting a balanced diet have no problem to cover their carnitine needs through a combination of dietary intake and internal carnitine biosynthesis (Giovannini *et al.*, 1991). But carnitine supplementation is essential for preterm infants who cannot yet biosynthesize the compound due to the lack of the last enzyme of the L-carnitine biosynthesis pathway (Borum and Bennet, 1986) and for people suffering from various types of carnitine deficiencies. The role of carnitine in many such pathologies has mostly been ascribed to its involvement in fatty acid metabolism (De Simone and Famularo, 1997). But nowadays, carnitine is used for the treatment of a growing spectrum of other, unrelated diseases, including AIDS and Alzheimer's disease (De Simone and Famularo, 1997), suggesting that carnitine's involvement in metabolic activities affects other levels of cellular activity. In this regard, it is known that in bacteria carnitine does not play a role in fatty acid metabolism, nor in glucose oxidation; but can be fully catabolized to be used as sole source of carbon or nitrogen, degraded to generate energy or metabolites, and can also act as an osmoprotectant (Rebouche and Seim, 1998).

Carnitine related metabolic activities in *S. cerevisiae* have been investigated by several groups (Van Roermund *et al.*, 1998; Van Roermund *et al.*, 1999; Swiegers *et al.*, 2001). These studies indicated that carnitine is not essential for growth in any of the studied conditions and that this yeast is unable to synthesize the compound. Nevertheless, several non-essential carnitine-dependent metabolic activities exist. As in other eukaryotes, carnitine is indeed involved in the transfer of activated acyl residues across the membranes of cellular organelles. In yeast, this involves in particular the transfer of activated acetyl residues from the peroxisome or the cytoplasm to the mitochondria. β-oxidation in this yeast is exclusively peroxisomal, and acetyl-CoA is generated solely in the peroxisomes when the yeast is grown on fatty acids as sole carbon source. However, acetyl-CoA can not be transferred across organellar membranes. The activated acetyl residue is therefore transferred to a molecule of carnitine in a reaction catalyzed by a carnitine-acetyl transferase (CAT). Acetyl-carnitine can be transported to the mitochondria, and crosses organellar membranes through a carnitine/acetyl-carnitine antiport translocase system. In the mitochondrion the reverse reaction takes place (transfer of the activated acetyl residue back to a molecule of free CoA-SH), and the free carnitine can be shuttled back to the peroxisome. The carnitine shuttle is not essential for growth since the requirement for this transfer can be by-passed by the glyoxylate cycle, a modified version of the tri-carboxylic acid (TCA) cycle. This cycle, which is at least in part peroxisomal,

integrates two molecules of acetyl-CoA and results in the net formation of a C_4-compound, succinate. Succinate can be transported to the mitochondria and serve as a carbon source to sustain growth. As a consequence, some glyoxylate cycle mutant strains, for example *cit2* strains, which are lacking peroxisomal citrate synthase activity, are dependent on the presence of extracellular carnitine for growth.

In the study presented here, *S. cerevisiae* is used as a model system to further assess the physiological roles of carnitine in eukaryotic organisms, and to in particular identify roles which may not be accounted for by the metabolic pathway described above. The fact that *S. cerevisiae* can not synthesize carnitine is advantageous for such a study since it allows to relate any observed physiological effect directly to the amount of extracellular carnitine provided in the growth substrate.

1.2 PROJECT AIMS

The multiplication of the use of L-carnitine and the suggestion that carnitine may play additional, unknown roles in cellular metabolism was at the origin of this project. These other metabolic properties may help to explain the general improvement observed for some patients under carnitine supplementation. Our research focused on investigating other possible properties of carnitine in yeast. More specifically, the project entailed:

(i) Conducting physiological and phenotypical tests to assess whether carnitine could play any other role in yeast than its involvement in fatty acid metabolism.
(ii) Determining if this new role may be specific to certain yeast species or strains or is of general relevance.
(iii) Identifying potential gene(s) which could be involved in such processes.

In addition the research project also required to contribute to the development of a method to quantify carnitine and its derivates. A fourth aim of the study therefore was

(iv) Developing an ES-MS/MS method for measurement of carnitine and its derivates.

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CHAPTER 2

LITERATURE REVIEW

Carnitine: Roles and Functions

LITERATURE REVIEW

2.1 INTRODUCTION

Carnitine was discovered at the beginning of the previous century (1905) by two independent research groups. The name "carnitine" derives from the Latin word *carnis* (meat), since Gulewistisch and Krimberg (1905) first isolated it from meat. This is also the reason why for many years carnitine was considered to be present only in vertebrate muscular tissue. Gulewistisch and Krimberg later proposed a chemical formula, $C_7H_{15}NO_3$, for carnitine. A second research group, headed by Kutscher (1905), reported the isolation of two similar compounds from meat, novain and oblitin. After several years of confusion, Krimberg (1907) demonstrated that novain was in fact carnitine and that oblitin was the dimethyl-ester of carnitine. He also proposed a molecular structure for carnitine (Structure I, Fig. 2.1; Krimberg, 1907) which was challenged by that of Engeland in 1909 (Structure II, Fig. 2.1; Engeland, 1909). The identification of γ-butyrobetaine as a product of carnitine reduction led Krimberg to justify the position of the hydroxyl group, whereas Engeland based his analysis on the formation of β-homobetaine as a product of carnitine calcium permanganate oxidation. Engeland conceded that the structure he proposed was based on a compound that was different from natural carnitine (Engeland, 1921).

Structure I Structure II

Figure 2.1 The structures of carnitine as proposed by Krimberg (1907) and Engeland (1909).

The isolation of carnitine from meat as published by Gulewistsch and Krimberg (1905) resulted in the development of several methods for the isolation of carnitine from natural products. However, it was the chemical synthesis of DL-carnitine (Rolett, 1910; Engeland, 1910) that eventually confirmed carnitine to be β-hydroxy-γ-trimethylammonium butyrate (Tomita and Sendju, 1927). Tomita and Sendju synthesized both isomers and reported that the levogyre isomer was the natural form (1927). This resulted in several methods with which the individual carnitine isomers could be synthesized (Carter and Bhattacharyya, 1953, Descamps *et al.*, 1954).

The discovery of the biological requirement for carnitine marks an important step in the understanding of the role of carnitine in metabolism. *Tenebrio molitor* was the first carnitine-dependent organism identified (Fraenkel and Blewett, 1947). The larvae of this yellow mealworm are not able to grow on a diet composed of glucose, casein, cholesterol, a salt mixture and nine types of vitamin B. It is, however, able to grow and survive with a

low supplementation of yeast or liver extract. Testing folic acid (vitamin B_c) as a possible factor responsible for overcoming the inability to grow showed that even if the growth is enhanced in the presence of folic acid, the larvae are rapidly dying despite being overweight. Concluding that an active principle must have been present in the yeast or liver extract, Fraenkel and co-workers named the unidentified survival factor vitamin B_T. They called it vitamin B for its similarity to folic acid which also allowed *Tenebrior molinor* to survive for a few days without carnitine. The T was an abbreviation for *Tenebrior* (Fraenkel *et al.*, 1948).

In 1952, Carter and co-workers definitively established the identity of vitamin B_T as carnitine through comparative analysis of physiological and derivative properties of pure vitamin B_T and carnitine extracted from liver and whey. Carnitine was isolated, purified and identified from liver as well as whey and quantified in meat extract. Even without investigating the activity of D-carnitine, it was determined that the racemic solution was less active than the natural isomer (Carter *et al.*, 1952). Carnitine was first considered as a vitamin-like molecule and it has been classified as a baby vitamin in recognition of its critical role during the growth of newborns, whose main source of energy is provided by oxidation of milk fatty acids. It has since also become apparent that carnitine is a compound universally present in most, if not all investigated forms of life such as bacteria, plants, fungi, invertebrates and vertebrates (Fraenkel and Friedman, 1957).

Initially, limited means to study carnitine and the absence of a sufficiently sensitive carnitine detection method were responsible for a lack of biochemical and functional knowledge on carnitine. However, the fact that some organisms showed a low carnitine requirement, while others contained relatively high concentrations was taken as evidence to suggest two different (and undetermined) levels of carnitine involvement in cellular metabolism.

In 1955, Fritz identified the first metabolic link suggesting a function for carnitine, the increase of fatty acid oxidation in liver and liver homogenates. In the same year, Friedman and Fraenkel discovered the reversible enzymatic conversion of carnitine to acetylcarnitine in the presence of acetyl-CoA.

The role of carnitine in the metabolism of fatty acids was confirmed by the observation that carnitine is able to enhance the transfer of long chain fatty acids into the mitochondrion for fatty acid oxidation. These discoveries led to the understanding of the role that carnitine can play as an acylcarrier by shuttling activated acyls into and through the mitochondrial matrix (Fritz, 1963). Little additional data were generated over a period of time, despite the fact that carnitine was no longer considered to be exclusively a meat compound. Research only intensified after the discovery of the first human carnitine deficiency in the early 1970's (DiMauro and DiMauro, 1973; Engel and Angelini, 1973). This research has revealed in more detail the critical role of carnitine in the transfer of activated acyl residues in various species. The major historical steps of carnitine-related research are summarized in Table 2.1.

Table 2.1 Overview of the main carnitine discoveries during the last century. Adapted from Claudio De Simone and Giuseppe Famularo, 1997.

Year	Discovery
1905	Carnitine is isolated from muscle (Gulewitsch *et al.*, 1905).
1927	The final chemical structure of carnitine is established (Tomita and Sendju, 1927)
1952	Carnitine is shown to be a vitamin (B_T) for a mealworm (Tenebrio molitor) (Carter *et al.*, 1952).
1955	Carnitine is shown to stimulate fatty acid oxidation in liver homogenates (Fritz, 1955).
	Reversible enzymatic acetylation of carnitine in liver is detected (Friedman and Fraenkel, 1955).
1961	Butyrobetaine is shown to be a carnitine precursor (Lindstedt and Lindstedt, 1961; Bremer, 1962a).
1962-63	Fatty acid esters of carnitine are shown to be intermediates in fatty acid oxidation (Bremer, 1962b; Bremer, 1962c; Fritz and Yue, 1963).
1965	High concentrations of carnitine are found in epididymis and sperm (Marquis and Fritz, 1965).
1966	CPT (carnitine palmitoyltransferase) is localized in the inner mitochondrial membrane and acyl-CoA synthetase in the outer mitochondrial membrane (Norum *et al.*, 1966; Yates *et al.*, 1966).
1970	Formation of branched chain acylcarnitines from branched chain amino acids is detected (Solberg and Bremer, 1970).
1971	Lysine is shown to be a precursor of carnitine (Horne *et al.*, 1971).
1973	Carnitine acyltransferases are detected in peroxisomes (Markwell *et al.*, 1973).
	Inborn errors in carnitine metabolism are found (DiMauro and DiMauro, 1973; Engel and Angelini, 1973).
1975	Carnitine translocase is demonstrated in mitochondria (Ramsay and Tubbs, 1975; Pande, 1975).
1977	Malonyl-CoA is shown to be an inhibitor of CPT-1 (McGarry *et al.*, 1978).
1980-81	Fatty acid oxidation is found to be less inhibited by malonyl-CoA in livers of fasted rats (Cook *et al.*, 1980; Bremer, 1981).
1987	CPT-1 is shown to be localized in the outer membrane of the mitochondria (Murthy and Pande, 1987).
1988-now	Several genes of carnitine acyltransferases and other enzymes involved in carnitine-dependent metabolic activities are cloned and their regulation studied

2.2 PHYSICAL AND CHEMICAL PROPERTIES OF CARNITINE

Carnitine is a small water-soluble molecule of seven carbon atoms, known as 3-carboxy-2-hydroxy-N,N,N-trimethyl-1-propanaminium, γ-amino-β-hydroxybutyric acid trimethyl-betaine, γ-trimethyl-β-hydroxybutyrobetaine or 3-hydroxy-4- (trimethylammonia) butanoate according to chemical nomenclature. The chemical formula of carnitine is $(CH_3)_3N^+CH_2CH(OH)CH_2COO^-$ with a molecular weight of 161.20 g/mol. Carnitine belongs to the betaine family and has been classified as vitamin B_T (Merck Index, 2001).

The L(-)form is the natural isomer and is found under different names and trademarks such as levocarnitine, vitamin B_T, Cardiogen, Carnitene, Carnitor, Carnum, Carrier, Miocor and Miotonal. The D-form is mainly a waste product of L-carnitine chemical synthesis. As such, the word carnitine almost always refers exclusively to L-carnitine. L-carnitine forms crystals in ethanol and acetone and has a melting point of 197-198°C. It is very hygroscopic,

soluble in water and in hot alcohol, practically insoluble in acetone, ether and benzene. $[\alpha]^{30}_D$ = -23.9° (c = 0.86 in water) (Merck Index, 2001).

D-carnitine crystals have a melting point of 210-212°C., and $[\alpha]_D$ = + 30.9°. This isomer is very soluble in water and alcohol and practically insoluble in acetone or ether. D-carnitine is not used in therapy, but the DL-form (hygroscopic crystalline solid, melting point 195-197°C) can be used as a gastric and pancreatic stimulant (Merck Index, 2001).

2.3 IMPORTANCE AND ROLE OF CARNITINE

2.3.1 CARNITINE IN HUMANS

In 1973, carnitine palmityltransferase deficiency was described in muscles and classified as a primary deficiency (DiMauro and DiMauro, 1973; Engel and Angelini, 1973), developing the interest for research on carnitine.

2.3.1.1 CARNITINE LEVELS

Mammals are not able to degrade carnitine, and only enzymes of some microorganisms, including some that are present in the gastrointestinal flora, can be responsible for its catabolism (Rebouche *et al.*, 1984; Seim *et al.*, 1985). Dietary carnitine is being absorbed by an active mechanism (Li *et al.*, 1992), but therapeutic doses are absorbed by passive diffusion. Microorganism flora in the large intestine catabolizes L-carnitine into trimethylamine oxide (8 to 89 % of total carnitine, eliminated in urine) and γ-butyrobetaine (0.1 to 8 % of total carnitine, excreted in feces) (Rebouche and Chenard, 1991).

Since L-carnitine is principally provided by dietary sources (red meat, poultry, fish, milk products), *de novo* synthesis is relatively low. As a consequence, absorption from the intestine and reabsorption from extra cellular fluid are mainly responsible for carnitine levels and its distribution (Fig. 2.2).

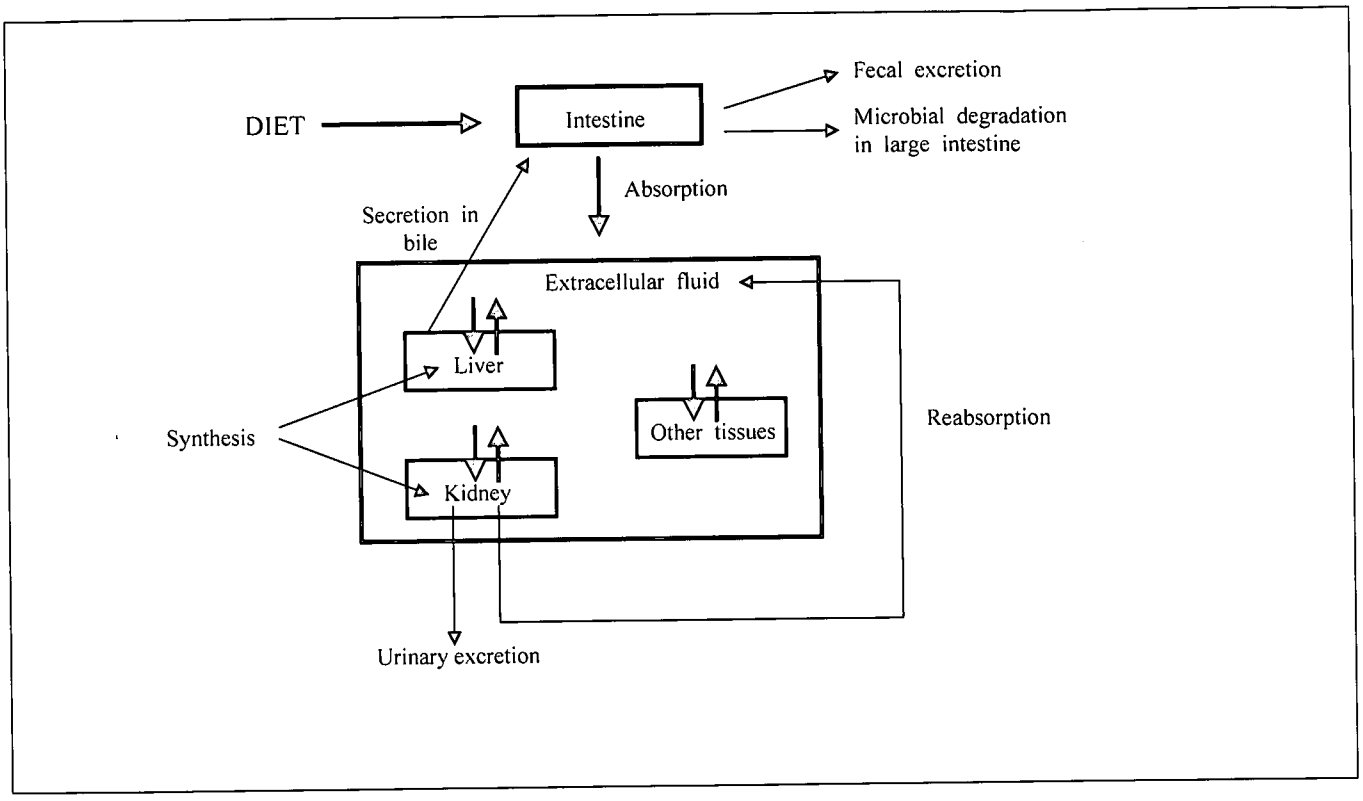

Figure 2.2 Scheme of carnitine homeostasis in mammals (adapted from Rebouche and Seim, 1998).

In humans, different forms of L-carnitine are found in the body, including free carnitine and esterified carnitine (acylcarnitine) with either short chain fatty acids (mainly acetylcarnitine) or with long chain fatty acids. Most of the carnitine is found in the skeletal muscle (3 mM) (Cederblad *et al.*, 1974). Although normal carnitine concentration in blood plasma is between 25-50 μM, most tissues contain a carnitine concentration 20 to 50 fold higher than that of blood plasma (Bremer, 1990; Carter *et al.*, 1995). Carnitine is also found in blood cells, especially in red blood cells (Borum *et al.*, 1985), and both plasma and blood cells are containing carnitine, short-chain acyl carnitine (mostly acetyl-carnitine) and long chain acyl carnitine (Borum, 1987).

After a rapid intestinal absorption (active and passive mechanism), carnitine is released into the systemic circulation where under normal conditions more than 90% of plasma carnitine levels are reabsorbed by the kidney (Rebouche and Mack, 1984). Carnitine threshold in plasma for carnitine excretion is estimated to be equivalent to plasma carnitine levels, suggesting that at normal physiological condition, plasma carnitine concentration is regulated or partly regulated by kidney carnitine reabsorption (Engel *et al.*, 1981; Rebouche and Paulson, 1986).

Even if most cells are able to import carnitine, as a carnitine gradient 10 to 100 fold is found between extracellular and intracellular concentration, skeletal muscle represents about 90% of body carnitine stores and 98% for skeletal muscles and heart (Kletzmayr *et al.*, 1999). This muscle concentration is being reduced by exercise which favors esterification of free carnitine (Lennon *et al.*, 1983). About 1.5% of the total body carnitine is found in liver and kidney and only 0.5% in the intracellular fluid (Table 2.2, Engel and

Rebouche, 1984). In the brain, carnitine content is low and its concentration appears to be affected by some neurotransmitters (Virmani *et al.*, 1995).

Table 2.2 Carnitine concentration in human tissues.

Tissue	Carnitine level (nmol/g wet weight)
Skeletal muscle	1140-3940
Myocardium	610-1300
Kidney	330-600
Liver	500-1000
Brain	500-1000

These differences in carnitine levels can be explained by the role of carnitine in fatty acid oxidation and energy production in muscles, as well as by carnitine biosynthesis and the influence of factors such as sex and other hormones, including glucagon and insulin (Bremer, 1990; Carter *et al.*, 1995), but also by carnitine turnover.

Carnitine turnover was approximately of 8 days in skeletal muscle and heart cells, of 11.6 hours in liver and kidney, of 1.13 hours in extracellular fluid and of 66 days in the whole body (Rebouche and Engel, 1984). It is also interesting to notice that plasma carnitine levels are varying with age, from 25 µmol/L during infancy - newborn carnitine synthesis is very low because of a low activity of γ-butyrobetaine hydrolase, but breast milk contains 40 to 70 µmol/L of carnitine - to 54 µmol/L in old age (Bremer, 1990; Carter *et al.*, 1995), and that male values are higher than female values, respectively 59.3±11.9 µM and 51.5±11.6 µM (Engel and Rebouche, 1984). This difference can probably be explained by the fact that males have higher muscle content as well as a higher basic metabolism. These differences in carnitine levels and distribution involve regulatory processes of carnitine homeostasis that have not been identified even if hormones may play a role.

2.3.1.2 *DE NOVO* SYNTHESIS

The pathway for *de novo* synthesis of carnitine is indicated in Fig 2.3. Carnitine *de novo* synthesis in human (and most other organisms) is depending on two essential amino acids, lysine and methionine, and involves various cofactors, including vitamin C, niacin, pyridoxal phosphate, 2-oxoglutarate, NAD^+ and iron (Haeckel *et al.*, 1990). Lysine is the precursor of carnitine biosynthesis. In the first step of biosynthesis, lysine has to be tri-methylated. In mammals, this methylation only occurs on peptide-bound lysine with S-adenosyl-L-methionine serving as a methyl donor. The tri-methylation of lysine is part of the specific posttranslational modification of certain proteins. Indeed, ε-N-trimethyllysine residues are present in many proteins such as histones, cytochome C, myosin and calmodulin (Paik and Kim, 1980). Trimethyllysine becomes available for further

biosynthesis after protein degradation and can be converted to γ-butyrobetaine in most tissues.

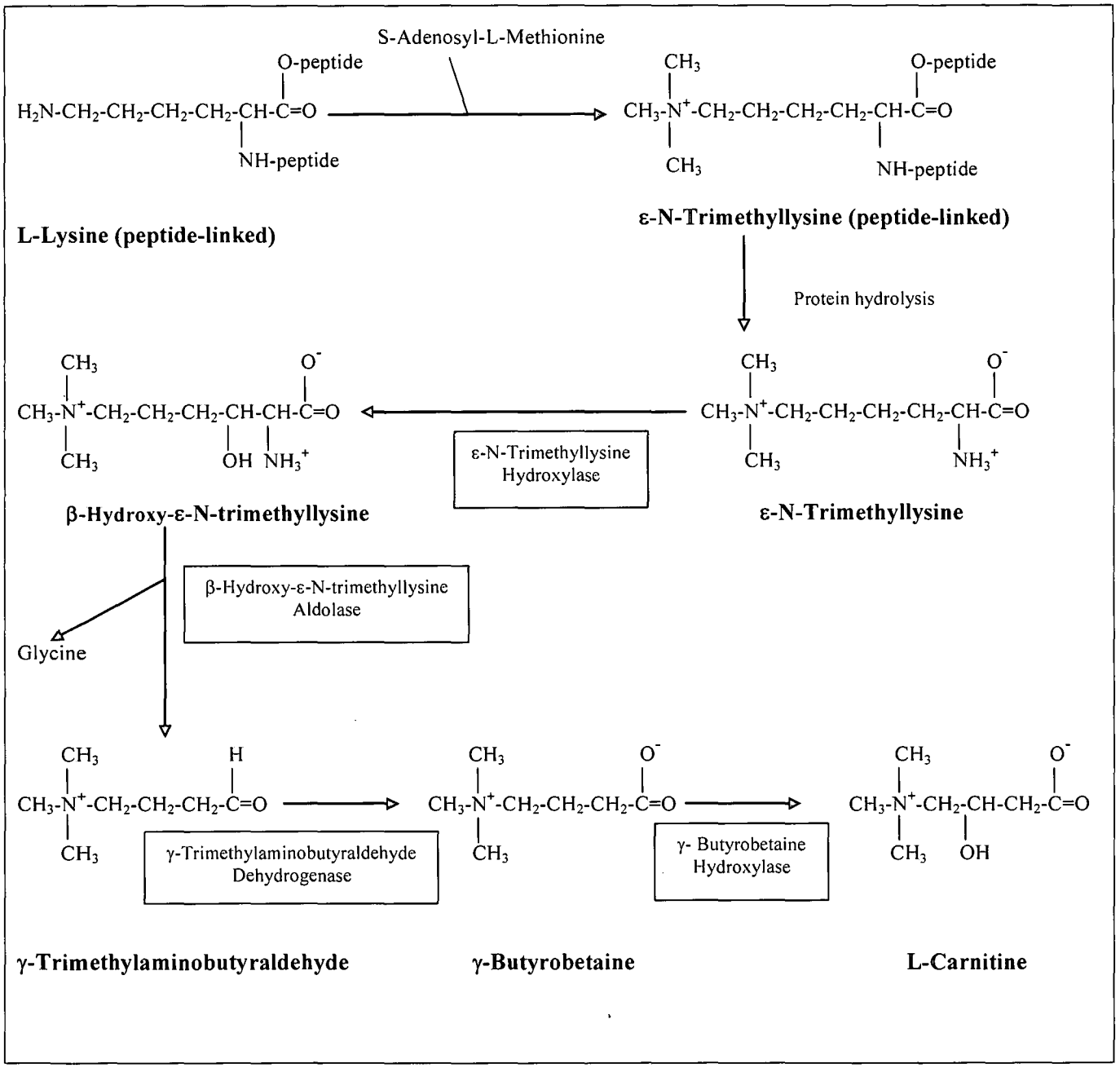

Figure 2.3 L-carnitine biosynthesis in eukaryotes. The carnitine carbon chain is provided by L-lysine whereas the methyl groups are provided by S-adenosyl-methionine (Rebouche, 1992a). Carnitine is mostly produced in the liver (although brain and kidney can also synthesize it) and is released into the blood circulation system (and so are acylcarnitines) from where it is imported in tissues. Its released into the systemic circulation is specifically ensured by liver and kidney.

The next reaction in the biosynthesis pathway is catalyzed by ε-N-trimethyl-L-lysine hydroxylase (E.C. 1.14.11.8), which requires α-ketoglutarate as a co-substrate. This enzyme is found in the mitochondria of cells present in various organs and tissues (liver,

kidney, heart, skeletal muscle and brain). In the next reaction, a serine hydroxymethyltransferase (E.C. 2.1.2.1) cleaves the β-hydroxy-ε-N-trimethyllysine to generate γ-trimethylaminobutyraldehyde and a molecule of glycine, a reaction that requires the presence of pyridoxal phosphate.

γ-Butyrobetaine is formed by the oxidation of this aldehyde, which is being hydroxylated by another hydroxylase (a dioxygenase), γ-butyrobetaine hydroxylase (E.C. 1.14.11.1), requiring α-ketoglutarate as co-substrate (Rebouche, 1991), to form carnitine.

2.3.1.3 REGULATION OF BIOSYNTHESIS

Despite the fact that hepatic γ-butyrobetaine hydroxylase activity is about 25% of the adults activity in newborns (Olson and Rebouche, 1987), and that this enzyme activity is not a limiting factor for carnitine synthesis (Rebouche, 1992a), the newborn's dependence for fatty acid oxidation from milk as a source of energy explains the classification of carnitine as a baby vitamin.

Studies show that it is likely that substrate availability and accessibility rather than enzyme activity are limiting the production of carnitine. In particular, it is likely that the amount of ε-N-trimethyl-L-lysine generated from protein turnover is responsible for limiting carnitine biosynthesis (Rebouche, 1992a).

The carnitine biosynthesis rate appears quite constant and is approximately of 2 μmol per kg of body weight per day. This would indicate that 30 to 50 % of total ε-N-trimethyl-L-lysine (provided by diet and endogenously synthesized) is being utilized for L-carnitine synthesis, whereas the excess is excreted in urine (Rebouche and Seim, 1998).

Depending on the kind of diet adopted by individuals, endogenous L-carnitine generation will vary. As the main source of carnitine is meat (beef, lamb, pork), omnivorous will have the majority of their daily requirement in L-carnitine provided by their diet, while strict vegetarian will be highly dependant on *de novo* synthesis (Lombard *et al.*, 1989).

2.3.1.4 CARNITINE ABSORPTION AND DISTRIBUTION

54 to 87 % of carnitine is being absorbed from the diet (Rebouche, 1992b; Rebouche *et al.*, 1984) and carnitine *de novo* synthesis is mainly ensured by liver tissue. Carnitine is then released into the blood circulation system (Fig. 2.2).

Carnitine absorption and distribution in mammals is ensured by two transporters, OCTN2 and $ATB^{0,+}$ (Nakanishi *et al.*, 2001). OCTN2 is a member of the organic cation transporters, and is widely expressed in human as well as in mammalians tissues. hOCTN2 refers to the human sodium-driven organic cation transporter 2. It is strongly expressed in kidney, skeletal muscles, heart and in the placenta but poorly expressed in the intestine (Taylor, 2001). The transporter is energized by a sodium gradient and membrane potential (Nakanishi *et al.*, 2001). A genetic defect in the hOCTN2 transporter can lead to a primary carnitine deficiency which will result in an impairment of fatty acid

oxidation and result in a skeletal and cardiac myopathy and encephalopathy (Seth *et al.*, 1999). In this case, carnitine is no longer actively accumulated in the heart and skeletal muscles but excreted in urine (Wu *et al.*, 1998; Tamai *et al.*, 1998).

OCTN2 is able to stereoselect carnitine, with a high affinity for L-carnitine (K_m= 4.8 ±0.3 μM) when compared to D-carnitine (K_m= 98.3 ±38.0 μM). It shows a comparable affinity for propionyl-carnitine and acetyl-carnitine than for L-carnitine (Wu *et al.*, 1999). It is also able to transport betaines and is inhibited by most of the drugs known to induce systemic carnitine deficiency, such as emetine (antibiotic), quinine and verapamil (ion channel blockers) (Wagner *et al.*, 2000).

$ATB^{0,+}$ is a novel carnitine transporter belonging to the neurotransmitter transporter gene family. It is a sodium- and chloride-coupled amino acid transporter, presenting a high affinity for cationic and neutral amino acids. It can shuttle amino acids e.g. glycine and proline, neurotransmitters like monoamines and γ-aminobutyrate and osmolytes such as taurine and betaine (Wagner *et al.*, 2000). $ATB^{0,+}$ presents a low affinity for L-carnitine and propionyl-carnitine (K_m between 0.6-0.9 mM) and an extremely low affinity for acetyl-L-carnitine (Taylor, 2001). The relative position of the carboxyl and N-group of the molecule appears to be of importance for the recognition in the substrate site (Wagner *et al.*, 2000)

$ATB^{0,+}$ is primarily found in the intestinal tract, in the colon and in the small intestine, in lungs and mammary gland (Taylor, 2001). Although its affinity for carnitine is lower than the affinity of the OCTN2 transporter, it is a more efficient carnitine transporter as its concentrative capacity can be energized by both the ionic transmembrane gradient of Na^+ and Cl^- and by membrane potential (Wagner *et al.*,2000).

OCTN2 and $ATB^{0,+}$ are both contributing to carnitine intestinal absorption in healthy adults but as $ATB^{0,+}$ is also shuttling amino acids, there is a competition for carnitine transport, especially in the proximal region of the intestine. In the case of the therapeutic use of carnitine, it is therefore recommended to ingest carnitine supplements on an empty stomach (Taylor, 2001).

$ATB^{0,+}$ can play a significant role for carnitine transport in patients suffering from an OCTN2 genetic defect.

2.3.1.5 FUNCTION OF L-CARNITINE

Carnitine is well known for its role in the regulation of fatty acid oxidation and energy metabolism, and has more recently proven to be an important factor regulating glucose oxidation. This last action may contribute to explain some of the benefits observed for carnitine supplementation in several diseases.

2.3.1.5.1 Mitochondrial fatty acid oxidation

The oxidation of long chain fatty acids in mammals is mitochondrial. Fatty acids are esterified with carnitine to be shuttled into the mitochondria as acylcarnitines. Long chain fatty acid oxidation therefore is highly carnitine dependent (Bremer, 1983).

Four enzymes are involved in this mechanism: Carnitine palmitoyltransferase I and II, carnitine translocase and carnitine acetyltransferases (Fig. 2.4).

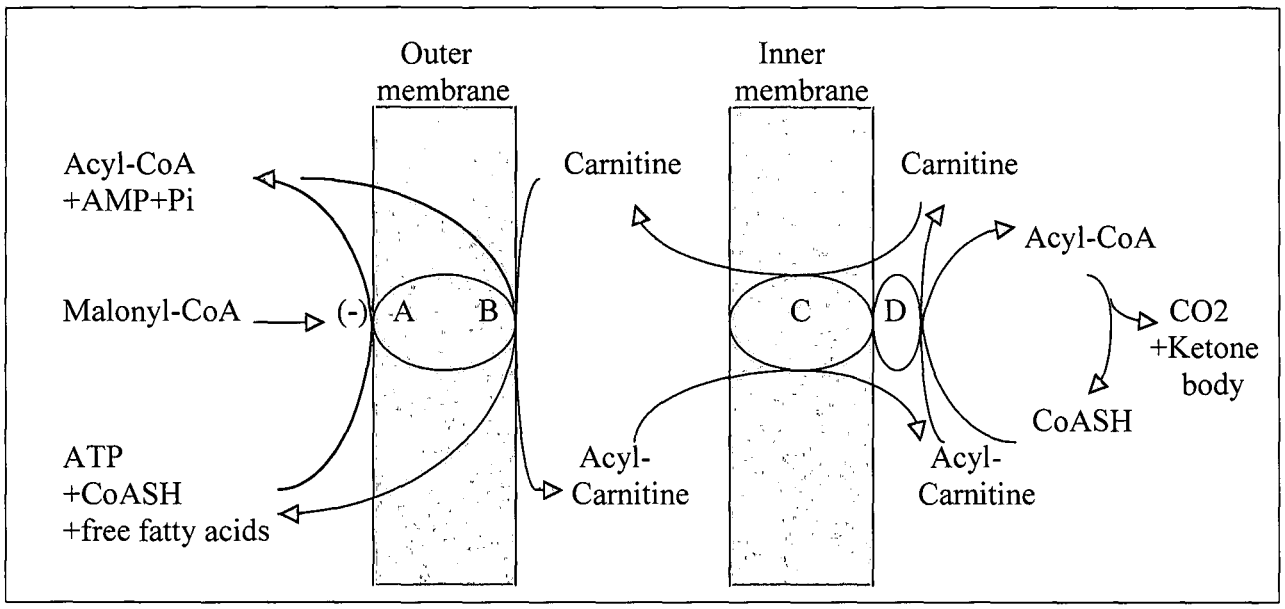

Figure 2.4 Organization of the carnitine dependent transport of activated fatty acids through mitochondria. AB: Carnitine palmitotransferase I (CPT-I) in the outer mitochondrial membrane with on the external surface the regulatory malonyl-CoA site (A) and its substrate site (B) on the inner surface (the outer membrane also contains the long chain acyl-CoA synthethase and the glycerophosphate acyltransferase competing with CPT-I for the acyl-CoA formed by the synthetase (Borrebaek, 1975).C: Carnitine/acylcarnitine translocase in the inner mitochondrial membrane. D: Carnitine palmitoyltransferase II (CPT-II) on the inner surface of the inner mitochodrial membrane. This is also the localization of the mitochondrial carnitine acetyltransferase.

Carnitine palmitoyltransferase I is located in the outer mitochondrial membrane and inhibited by malonyl-CoA (a fatty acid synthesis intermediate) and carnitine palmitoyltransferase II, is in the inner membrane facing the matrix. These two enzymes have a very broad activity, reacting with fatty acids varying in length from C_6 to C_{22}, and assure the reversible transfer of medium-chain and long-chain acyl-CoAs and carnitine to medium-chain and long-chain acylcarnitines (Bremer, 1990). The carnitine acetyltransferases are situated in the inner mitochondrial membrane, react preferably with acetyl- and propionyl-CoA, and are particularly active in heart and muscles (Bremer, 1990). Carnitine translocase is located in the inner mitochondrion membrane and represents an antiport system of acylcarnitines/carnitine across this membrane. Carnitine translocase, carnitine palmitoyl-transferase I and II are responsible for the transfer of free

long chain fatty acids that are activated to acyl-CoA in the outer mitochondrion membrane (or in the endoplasmic reticulum), into the mitochondrial matrix where they can be oxidized by the β-oxidation enzymes (Lombard *et al.*,1989).

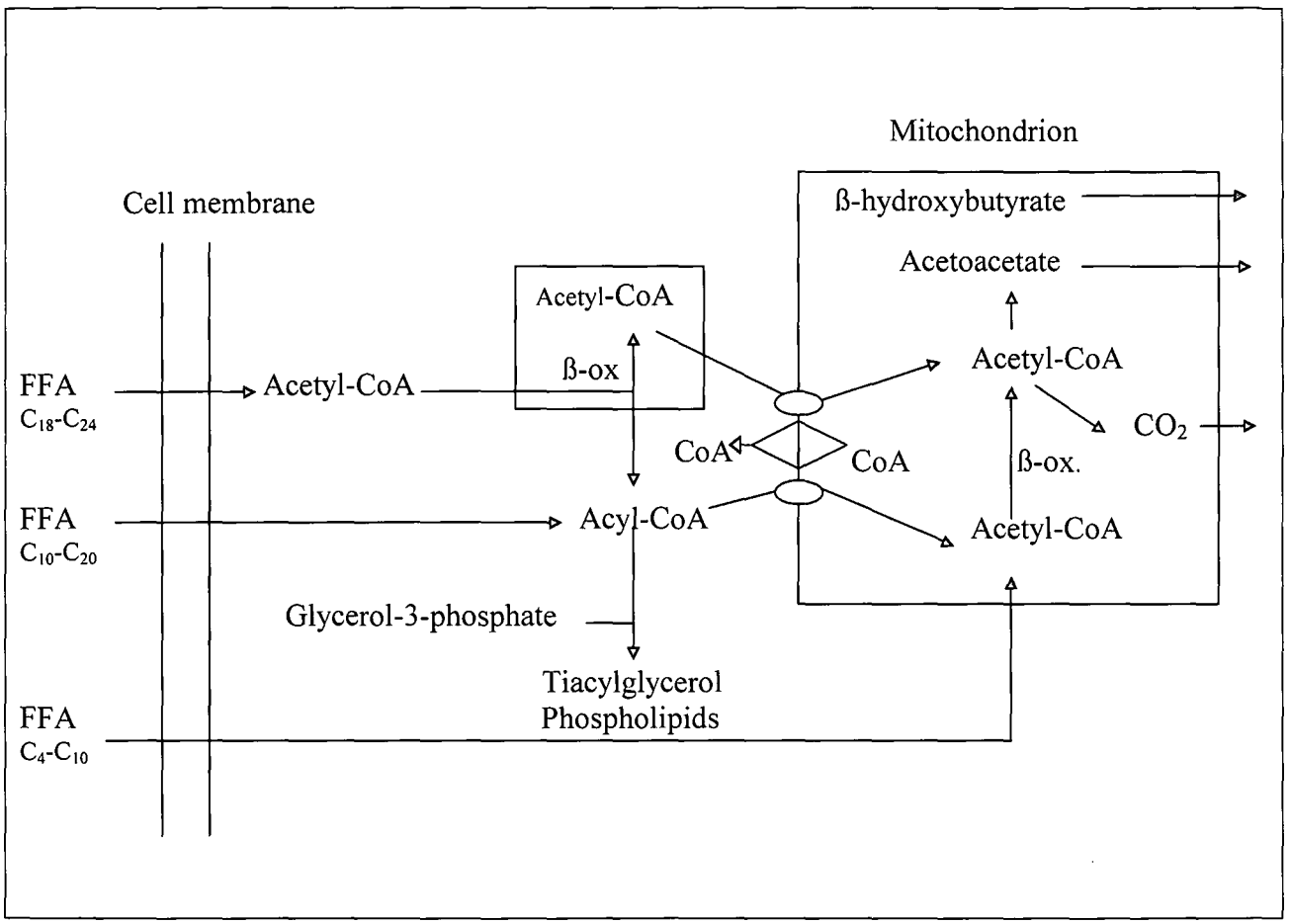

Figure 2.5 Fatty acid metabolism in liver. Free long chain fatty acids (FFA) are activated to acyl-CoA in the endoplasmic reticulum or in outer mitochondrial membrane. Mitochondrial circles represent the carnitine-dependent shuttle. B-ox: β-oxidation.

In fact in the cell, different specific carnitine acyltransferases (not all well known and localized) of varying chain length of fatty acids, are forming acylcarnitines (Bieber, 1988):

Acyl-CoA + carnitine ⇌ acylcarnitine + CoASH

Carnitine acylation is a reversible process and includes acylcarnitines synthesis from acetate (C_2) to eructate (C_{22}) (Bremer, 1983).

In mammals, peroxisomal fatty acid oxidation, contrarily to the mitochondrial one, is not dependent on carnitine – despite the presence of acetyl- and acyltransferases (Alkonyi *et al.*, 1975) – as an acyl-CoA carrier is present in the peroxisomal membranes (Appelkvist and Dallner, 1980; Osmundsen and Neat, 1979). In animals, these peroxisomal and mitochondrial acetyltransferases are very similar in structure and could hypothetically be encoded by the same gene and formed by alternative splicing (DiDonato and Finocchiaro, 1994).

Long and very long-chain fatty acids (up to C_{22}-C_{24}) are being partially oxidized in the peroxisome (Penn *et al.*, 1981) through a few β-oxidation cycles (Helms *et al.*, 1990; Sulkers *et al.*, 1990). These newly shortened acyl-CoA could then be associated with carnitine to form acylcarnitines, which would then be transferred to the mitochondria and be fully catabolized (Rebouche, 1992a) (Fig. 2.5). However, such action of carnitine acyltransferases has not yet been demonstrated.

2.3.1.5.2 Glucose oxidation

D-glucose is the preferred energy source of many organisms and has a key role in energy metabolism. It can be fully oxidized to pyruvate through glycolysis, can be stored in the cell as glycogen, or oxidized to pentose though the pentose phosphate pathway. It is not only a source of energy but also a precursor of various metabolic materials for biosynthesis reactions.

Acyl-CoA and short chain-CoA esters, including acetyl-CoA, can accumulate in the mitochondrial matrix, resulting in an inhibition of mitochondrial dehydrogenases (i.e. pyruvate dehydrogenase) leading to a decrease of mitochondrial oxidation of all the substrate and therefore to a decrease of mitochondrial energy production. In the same way, an increase of glucose oxidation may lead to lactate production with a risk of acidosis and of some functional alteration (i.e. impairment of muscle contractibility).

Carnitine, carnitine acetyltransferase and carnitine translocase, by forming and shuttling acetyl-CoA and short chain acylcarnitine from the matrix to the cytosol, can provide relieve from such accumulation. Carnitine is able either to relieve the inhibition of the pyruvate dehydrogenase complex or to simply enhance it in order to stimulate glucose oxidation. Because of this buffering role, CoASH will be available for other metabolic pathway. Excess carnitine esters can cross the mitochondrial membrane and be excreted in the urine (Fig 2.5 and 2.6).

Carbohydrate oxidation and fatty acids oxidation could be linked by a hypothetic role of carnitine as inhibitor of fatty acids oxidation. When cytosolic acetyl-CoA concentration increases, resulting in more substrate for acetylcarboxylase, malonyl-CoA molecules are being produced. As malonyl-CoA is an inhibitor of CPT-I, fatty acids oxidation is reduced. The heart for instance would then be able to have a more optimal supply of acetyl-CoA for the TCA cycle (Saddik *et al.*, 1993).

2.3.1.6 Carnitine dysfunctions and deficiencies

Great advances have been made since the discovery of the first fatty acid disorder due to carnitine deficiency and since several impairments of carnitine function have been described. These impairments and disorders may result in low levels of carnitine or some carnitine dysfunction.

As the energy supply for a prolonged muscle activity is provided by fatty acid oxidation, lipid storage myopathies are commonly found in carnitine deficiency and insufficiency. Lipid storage myopathies are associated with low levels of carnitine in skeletal muscles, in other tissues (i.e. liver and heart) and in the plasma. It is also characterized by muscle weakness; as well as central nervous system, heart and liver defects (Broquist, 1998).

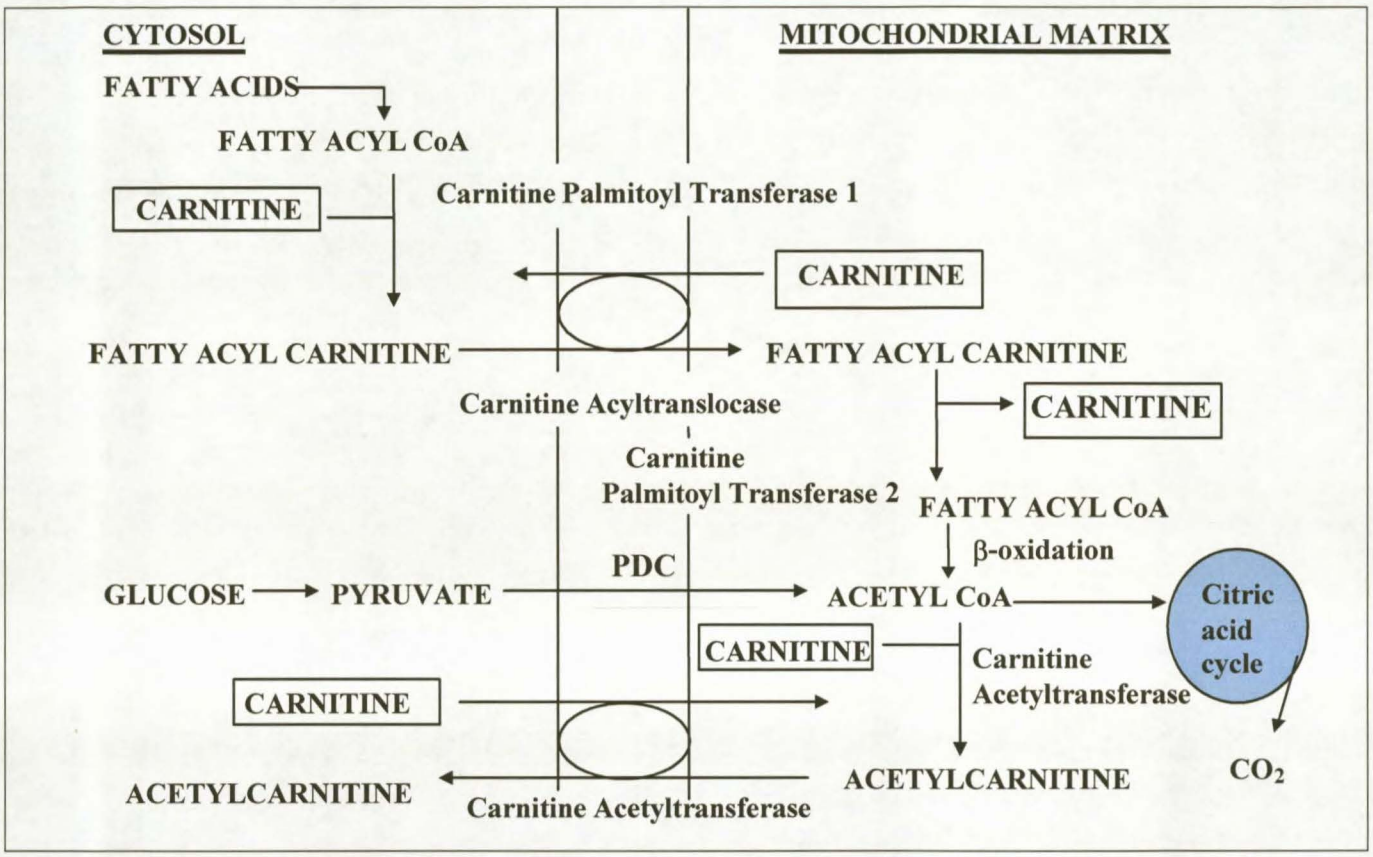

Figure 2.6 Sites of activity of L-carnitine in the heart. PDC: pyruvate dehydrogenase complex (adapted from Carnitine Today, 1997).

Low levels of carnitine can also be explained by a reduced carnitine biosynthesis capacity. This may be due to the limited availability of precursors or cofactors, to the dysfunction of organs in which biosynthesis takes place, to the direct impairment in the biosynthetic pathway (Rudman *et al.*, 1977), or to carnitine transport defects. In some lipid storage myopathies, normal carnitine enzyme as well as fatty acid oxidation systems are being observed, and the dysfunction appears due to an alteration of carnitine transport (Broquist, 1998).

Primary carnitine deficiency is mainly characterized by a defect in carnitine transport and by low levels of free carnitine in affected tissues, whereas secondary carnitine deficiency is characterized by an over production of CoA thioesters (acyls-CoA). Primary carnitine deficiency is defined as "a decrease of intracellular carnitine content that impairs fatty acid oxidation and that is not associated with another identifiable systemic illness that might deplete tissue carnitine stores" (Roe and Coates, 1989). The criteria for this condition are (1) a severe reduction of plasma or tissue carnitine levels, (2) evidence that the low

carnitine levels impair fatty acid oxidation, (3) correction of the disorder when carnitine levels are restored, and (4) absence of other primary defects in fatty acid oxidation (Treem *et al.*, 1988). Depending on the tissue distribution of the low carnitine content, primary carnitine deficiency can be divided into systemic or muscular carnitine deficiency. In the *systemic* form, there is a profound reduction of carnitine in the plasma and also in the affected tissues, whereas in the *muscular* form the low content is restricted to muscles" (Pons and DeVito, 1995).The treatment for this primary deficiency is a carnitine supplementation at a dose of 100 to 200 mg/kg of body weight/day (Treem *et al.*, 1988).

Secondary carnitine deficiency "which manifests with a decrease in the levels of carnitine in plasma or tissues, may be associated with genetically determined metabolic errors, acquired medical conditions, or iatrogenic states" (De Vito and Tein, 1990). Carnitine deficiency is an associated phenomenon of a large number of metabolic disorders. The most characteristic, representative causes of secondary carnitine deficiency are metabolic disorders associated with impaired oxidation of acyl-CoA intermediates in the mitochondria. These include fatty oxidation disorders and amino acid oxidation defects (Treem *et al.*, 1988). Most of secondary carnitine deficiency are lethal and include neonates' sudden death and spontaneous abortions.

Up to now, more than a hundred cases of this disease have been described (Zierz, 1994). In the case of fatty acid oxidation defect, it is important for the patient to have a high-carbohydrate and low fat diet, to take meals at regular and frequent intervals and to avoid fasting. A daily carnitine (riboflavine or glycine) supplementation will also be required (Treem *et al.*, 1988).

2.3.2 CARNITINE IN YEASTS

Yeasts belong to a group of unicellular fungi of the *Hemiascomycetae* and to the *Ascomycota*. They occur usually as single cells, but may under certain conditions be found as group or chains of cells. Although yeast is an eukaryotic organism, the yeast genome is very small ($1.4.10^7$bp), about 200 times smaller than the average mammalian genome ($3,5.10^9$bp), and the biggest yeast chromosome is about 100 times smaller than the average human chromosome.

Nevertheless, yeast cellular organization is similar to more complex organism, and many yeast proteins are structurally and functionally similar to their mammalian equivalent. Yeast is therefore often used to study eukaryotic gene function, expression, regulation and cell physiology. It can be more easily grown and manipulated than more complex organisms, and fundamental research that has been carried out on yeast have helped elucidate many general scientific concepts of cellular physiology, genetic regulation and metabolic pathways. Among yeasts, the baker's yeast *Saccharomyces cerevisiae* has been favored as a simplified model for higher eukaryotic cells.

2.3.2.1 L-carnitine biosynthesis in yeast

Most yeast species appear to be able to synthesize carnitine from lysine. It appears that some yeasts are able to trimethylate free lysine using S-adenosylmethionine as methyl donor, whereas in mammals, only peptide-bound lysine can be trimethylated (Bieber, 1988). However, data from van Roermund *et al.* (1999) and Swiegers *et al.* (2001) showed that *S. cerevisiae* is not able to neo-synthesize carnitine. Yeasts have not been shown to be able to catabolize carnitine.

2.3.2.2 Role of L-carnitine in yeast

2.3.2.2.1 Fatty acid metabolism in yeast

Contrary to mammals, β-oxidation of fatty acids in yeast is peroxisomal (Kunau *et al.*, 1995). The fact that mammalian cells require β-oxidation enzymes both for the mitochondria and peroxisomes makes *S. cerevisiae* a suitable model for studies on peroxisomal fatty acids oxidation when experimentation on higher eukaryotic cells becomes too difficult. Results of fundamental studies on yeast peroxisomes and peroxisomal fatty acid β-oxidation can be related to the human inherited disease of peroxisomal β-oxidation impairment *e.g.* X-linked adrenoleucodystrophy (Wanders *et al.*, 1995).

2.3.2.2.2 Origin, activation and distribution of fatty acids in yeast

Fatty acids in yeast can have three origins. They can be biosynthesized by fatty acid synthetase (FAS), be taken up from the external cell environment (exogenous fatty acids) or be provided from storage (triacylglycerols, phospholipids, and sterol esters). To be metabolized, they require activation as acyl-CoA esters and can then be used either for further lipid synthesis, acylation of proteins, be elongated or desaturated, or produce energy through the β-oxidation catabolic cycle (Daum *et al.*, 1998). Long chain fatty acids (principally palmitoyl and stearol) are converted to long chain fatty acyl-CoA (palmitoyl-CoA and stearol-CoA) by an acetyl-CoA carboxilase (Acc1p) (Hettema and Tabak, 2000). These long chain acyl-CoA esters can be used by *S. cerevisiae* as sole source of carbon and energy.

Fat1p has been shown to be involved in oleate uptake and Δ*fat1* cells show some disturbance in the metabolism of fatty acids and in fatty acid uptake (Faergeman *et al.*, 1997). The *FAT1* gene encodes a very long-chain fatty acyl-CoA synthetase, and, with two other proteins, Faa1p or Faa4p, may form a complex that imports and activates exogenous fatty acids (Choi and Martin, 1999; Watkins *et al.*, 1998; Zou *et al.* 2002, 2003) and regulates their accessibility prior to metabolization. Fat1p is partially associated with peroxisomes and the endoplasmic reticulum (Choi and Martin, 1999; Watkins *et al.*, 1998) and could also play a role in fatty acids uptake when fatty acids synthesis is compromised. According to Klein and co-workers (1971), acyl-CoA synthetase could be involved in a

"vectorial acylation" process: their role in uptake could be to convert these fatty acids that are membrane permeable to acyl-CoA esters impermeable to membranes.

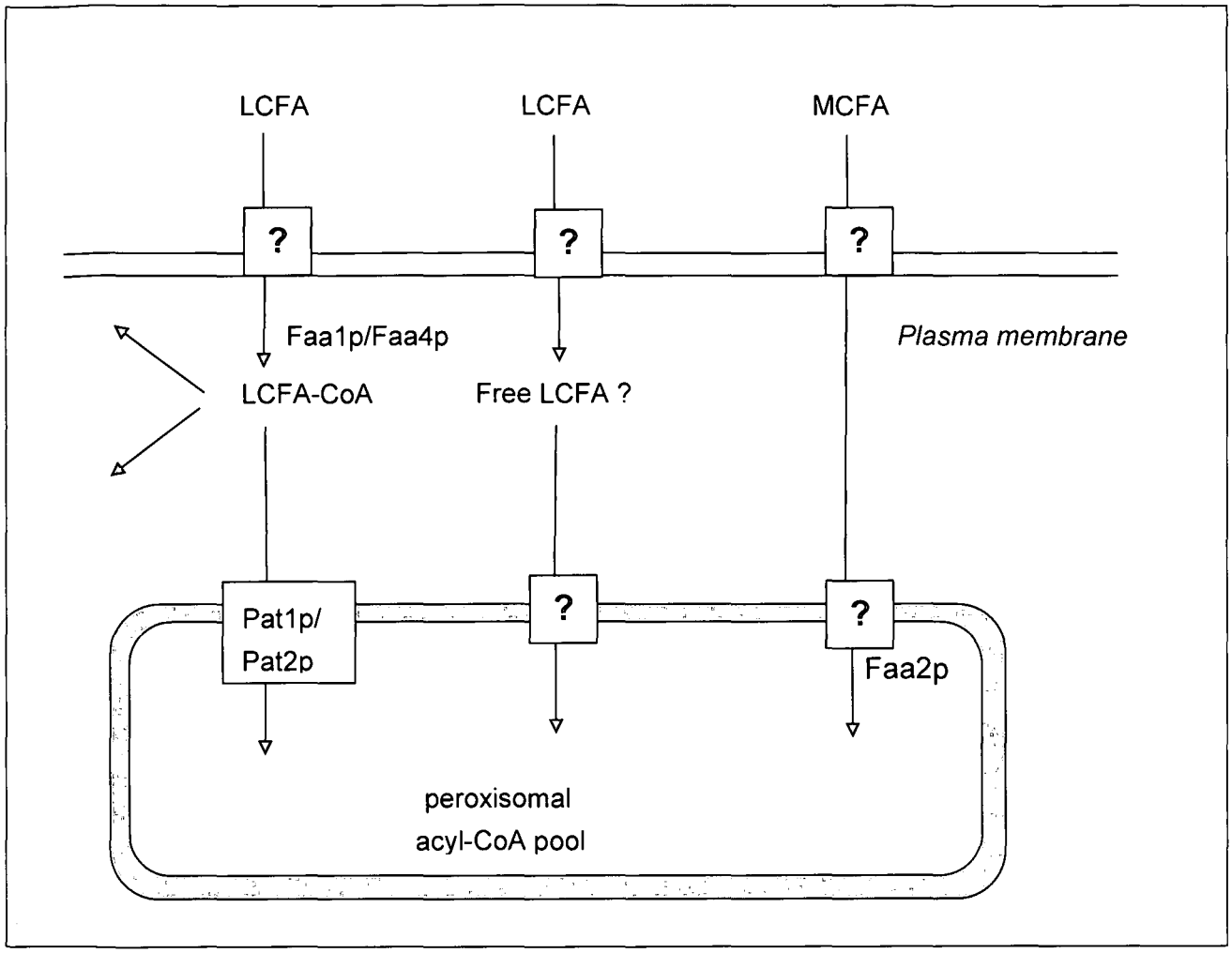

Figure 2.7 Schematization of fatty acids uptake by *S. cerevisiae*, activation and transport into peroxisomes (adapted from Hettema and Tabak, 2000).

In total, six open reading frames (ORFs) showing amino acids sequence homology to acyl-CoA synthetase are present in the *S. cerevisiae* genome (Knoll *et al.*, 1994). *In vitro*, acyl-CoA synthetase activity has been demonstrated for three of them (Faa1p, Faa2p and Faa3P). Faa1p and Faa4p are necessary to import long chain fatty acids and for their metabolization (Johnson *et al.*, 1994a). Faa2p is a peroxisomal enzyme required for medium-chain fatty acid β-oxidation (Hettema *et al.*, 1996). The role of Faa3p and Fat2p in fatty acids metabolism *in vivo* however has not been clarified (Johnson *et al.*, 1994b; Blobel and Erdman, 1996).

Medium chain fatty acids (<12C) either diffuse through the peroxisomal membrane or are transported by a protein prior to their activation as acyl-CoA by Faa2p (Elgersma and Tabak, 1995), as medium chain fatty acid-CoA synthesize activity is mainly peroxisomal (Hettema *et al.*, 1996).

Long chain fatty acids are principally being activated in the cymosely before import into the peroxisomes. This transport is ensured by an ABC transporter complex, with Pat1p and Pat2p (also named Pxa1p and Pxa2p) containing an ATP binding cassette (Elgersma and Tabak, 1995). Elgersma and Tabak (1995) suggested that Pat1p/Pat2p might be transporting either fatty acid-CoA esters or fatty acid bound to carnitine. However, this heterodimeric complex is only required for activated long chain fatty acids, as β-oxidation of long chain fatty acid activated inside peroxisomes is not depending on Pat1p/Pat2p (Hettema and Tabak, 2000). It is probable that long chain fatty acids (C16-C18) would preferably be activated in the cytoplasm and transported via the Pat1p/Pat2 complex (Fig. 2.7; Hettema and Tabak, 2000).

Contrary to mammals, *S. cerevisiae* shows no carnitine palmitoyl-transferase (CPT) activity (Hettema and Tabak, 2000), and the transport of fatty acids to the peroxisomes for β-oxidation is not carnitine dependent and is not similar to the long chain fatty acids (LCFAs) transport into mammalian mitochondria where LCFAs are activated to acyl-CoA and shuttled as carnitine esters.

When no other preferred carbon source is available, fatty acids are transported into the peroxisome to be degraded via β-oxidation processes to yield acetyl-CoA. Energy production from acetyl-CoA can only occur in the mitochondria, and the activated acetyl residue or some other usable intermediate has to be transferred to this organelle. The transfer of activated acetyl residues is normally ensured through the carnitine shuttle as described below. However, in the absence of carnitine, cells can by-pass the requirement for the shuttle by using the glycoxylate cycle. Two molecules of acetyl-CoA are integrated into each cycle, which leads to the formation of a C_4 dicarboxylic acid (succinate) which can be transported into the mitochondrion probably due to the activity of the hypothetical dicarboxylate carrier Acr1p (Palmieri *et al.*, 1997) (Fig. 2.8).

2.3.2.2.3 Genes involved in carnitine fatty acid metabolism of *S. cerevisiae*

The end product of fatty acid oxidation is acetyl-CoA. Once produced, these molecules need to be transported to the mitochondria via the cytoplasm so as to complete their degradation to H_2O and CO_2 and provide energy (Bieber, 1988; Osmunsden *et al.*, 1991; Reddy and Mannnaerts, 1994; Palmieri *et al.*, 1997). NADH produced during β-oxidation must also be reoxidized to ensure continuous β-oxidation.

The transfer of the activated acetyl residues from acetyl-CoA to carnitine is catalyzed by carnitine acetyltransferases (CATs). Three genes encoding such enzymes have been identified in *S. cerevisiae* (Van Roermund *et al.*, 1999; Swiegers *et al.*, 2001). The peroxisomal and mitochondrial carnitine acetyltransferases (pCat2p and mCat2p) are encoded by a single gene, *CAT2* (Elgersma *et al.*, 1995). When yeast cells are grown on oleic acid, Cat2p is responsible for more than 95% of the acetylcarnitine transferase activity (Kispal *et al.*, 1993). The gene is induced by ethanol and repressed by galactose.

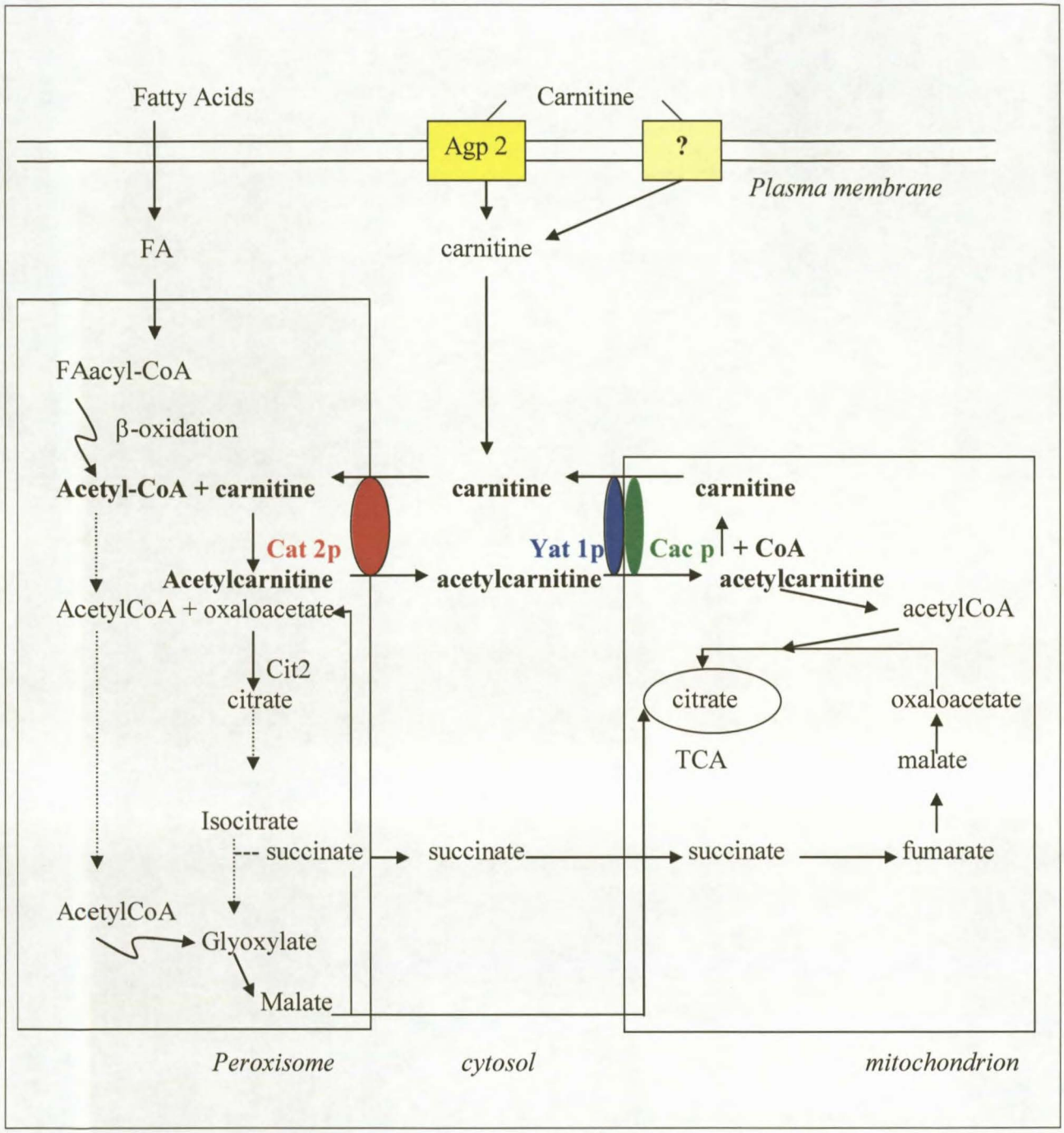

Figure 2.8 Fatty acids pathway in yeast. CAT: carnitine acyltransferase; *CIT2*: citrate synthetase gene (encoding the first enzyme of the glyoxylate cycle).Van Roermund and co-workers (1999) found that 20μM of L-carnitine was enough for a *CIT2* deleted strain for *S. cerevisiae* to grow normally on oleic acid media, suggesting that only a few molecules of carnitine are required for the acetyl-CoA shuttle and that *S. cerevisiae* is not able to synthesize carnitine *de novo* as yeast cells were not able to grow in these conditions without an L-carnitine supplementation.

The other two carnitine acetyl transferases are encoded by *YAT1* and *YAT2*. Yat1p has been shown to be associated with the outer mitochondrial membrane, while Yat2p appears to be cytoplasmic. All three enzymes are essential for the carnitine shuttle, since the corresponding mutants are unable to grow on fatty acids in the absence of the glyoxylate cycle. Indeed, Δ*cat2*Δ*cit2*, Δ*yat1*Δ*cit2* and Δ*yat2*Δ*cit2* (*CIT2* encoding the peroxisomal

citrate synthetase, the first enzyme in the glyoxylate cycle) double mutant strains can not grow on oleate as β-oxidation products from peroxisomes are not shuttled (Swiegers *et al.*, 2001). However, the specific roles of Yat1p and Yat2p have not been clearly defined.

Another gene required for the proper function of carnitine-dependent metabolic activities is the plasma membrane carnitine transporter, encoded by *AGP2* (*YBR132c*). The protein, Agp2p (596 AA, 12 potential transmembrane domains) belongs to the Major Facilitator Super family (MFS) of plasma membrane transporters (André, 1995) and is classified as a general amino acid permease which also allows carnitine uptake (van Roermund *et al.*, 1999).

CRC1 (*YOR100c*) encodes a translocase, member of the Mitochondrial Carrier Family (MCF), and is located in the inner mitochondrial membrane. The gene is responsible for the translocation of acetyl-carnitine into the mitochondria, and the export of free carnitine in an antiport system.

This list of genes encoding carnitine-related activities in the *S. cerevisiae* fatty acid metabolism is non-exhaustive. So far no carnitine peroxisomal translocase has been reported. It might be that, similar to the *CAT2* gene encoding both peroxisomal and mitochondrial carnitine acetyltransferase, *CRC1* encodes the carnitine acylcarnitine translocase for both the mitochondrion and peroxisome. (van Roermund *et al.*, 1999). Moreover, as Agp2p does not have any similarity to human carnitine transporter OCTN2, the existence of a specific yeast carnitine transporter responsible for carnitine uptake remains a possibility.

2.3.3 CARNITINE IN BACTERIA

Carnitine is ubiquitous and is also found in bacteria. Contrary to the role of carnitine in eukaryotes, no involvement of carnitine in the fatty acid metabolism of bacteria could be demonstrated to date (Kunau *et al.*, 1995; Rebouche and Seim, 1998). However, carnitine has been shown to act as an osmoprotectant, similar to betaine or proline (Kunau *et al.*, 1995), but its exact physiological role in bacteria remains unclear.

2.3.3.1 Carnitine metabolism in bacteria

Of all bacterial species, the carnitine metabolism of *Enterobacteriaceae* received the most attention. As part of the fauna of the gastrointestinal tract, these bacteria were shown to play an important role in carnitine deficiencies. Seim *et al.* (1982c) demonstrated that *Enterobacteriaceae* can decrease the levels of carnitine that are available from the diet and affect the levels of carnitine reabsorption when carnitine levels are already critical (Seim *et al.*, 1982c). Carnitine and crotonobetaine were also found to stimulate the anaerobic growth of *Enterobacteriaceae* (Seim *et al.*, 1985). These bacteria do not use carnitine as carbon or nitrogen source but are able, under anaerobic conditions, to catabolize L-carnitine and crotonobetaine into γ-butyrobetaine (Seim *et al.*, 1982c). L-

carnitine metabolism has also been studied in some *Proteus spp.* which were cultivated aerobically since these genera were not able to grow in anaerobic conditions. It was observed that *Proteus spp.* present the same carnitine metabolism than other *Enterobacteria*.

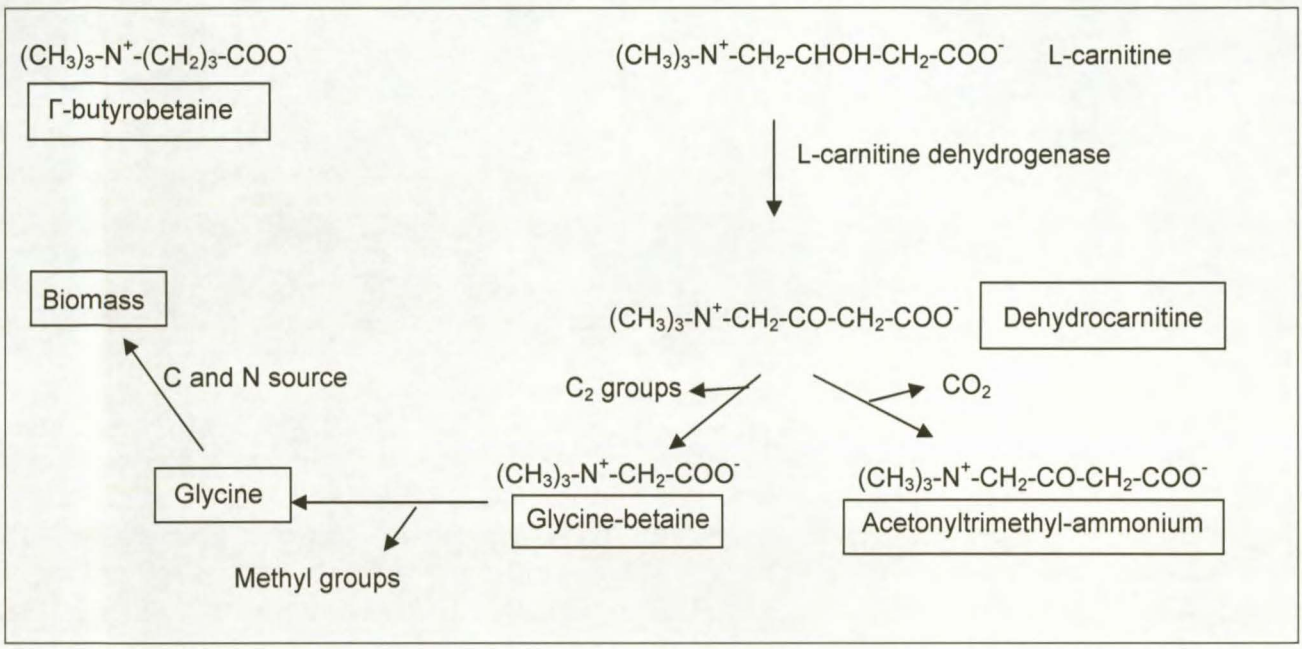

Figure 2.9 Aerobic degradation of L-carnitine by bacteria using L-carnitine as sole source of carbon and nitrogen. L-carnitine and 3-dehydrocarnitine are inducing the activity of L-carnitine-dehydrogenease (pathway from Rebouche and Seim, 1998).

Carnitine and crotonobetaine can induce carnitine metabolic enzymes and consequently its transformation to γ-butyrobetaine which cannot be exported out of the cell (Jung *et al.*, 1990). γ-Butyrobetaine therefore accumulates in the cell and represses the crotonobetaine reductase which favors L-carnitine synthesis instead of its degradation. The same effect was observed in other bacteria for which anaerobic carnitine catabolism reversed to an anabolism under aerobic conditions.

A different carnitine metabolism was reported for other bacterial species. In *Pseudomonas aeruginosa*, L-carnitine stimulates its own uptake whereas its oxidative degradation can be induced by both L-carnitine and crotonobetaine (Aurichet *et al.*, 1967; Kleber and Aurich, 1968).

These examples exemplify three major metabolic uses of carnitine in bacteria.

2.3.3.1.1 L-carnitine as sole source of carbon and/or nitrogen

Under anaerobic conditions, *Pseudomonas spp.* are able to use carnitine both as the sole source of energy and as carbon and nitrogen source (Rebouche and Seim, 1998). L-carnitine is oxidized to 3-dehydro-carnitine by a L-carnitine-dehydrogenase (EC:1.1.1.108; Fig. 2.9). This enzyme was also found in other genera such as Agrobacterium (Hanschmann *et al.*, 1996). *Acinetobacter spp.* use L-carnitine as sole source of carbon only (Kleber *et al.*, 1977; Seim *et al.*, 1982b).

2.3.3.1.2 L-carnitine metabolism for trimethylamine production

Some other species such as *Serratia marcescens* (Unemoto et al., 1966) and *Acinetobacter calcoacerticus* (Kleber *et al.*, 1977; Mancinelli *et al.*, 1995; Seim *et al.*, 1982b; Seim *et al.*, 1982a) are able to cleave L-carnitine or a racemic mixture of carnitine with variations at the fourth carbon to produce trimethylamine and malate (Fig. 2.10).

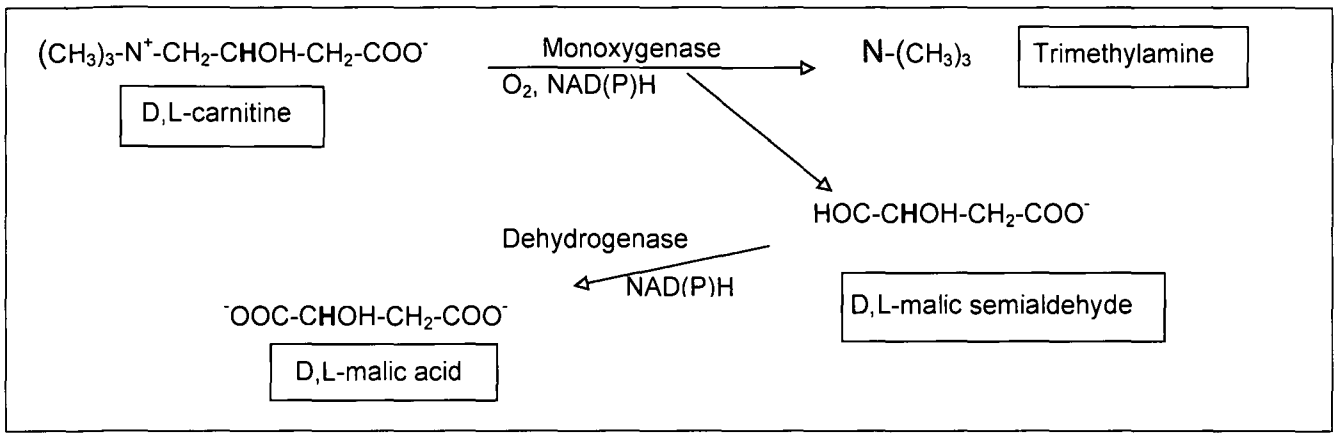

Figure 2.10 L-carnitine metabolism for trimethylamine production in bacteria. Trymethylamine formation by aerobic cleavage of the C_4 bond of carnitine (pathway from Rebouche and Seim, 1998).

2.3.3.1.3. Reduction of carnitine to γ-butyrobetaine

In the Enterobacteria, L-carnitine reduction was shown to be induced by L-carnitine and crotonobetaine, derepressed by fumarate, repressed by glucose, and inhibited by γ-butyrobetaine (but no other betaine-like molecules), and nitrate. Nitrate also represses the synthesis of L-carnitine metabolic enzymes (Seim *et al.*, 1982c).

It has been postulated (Seim *et al.*, 1982c) that during the anaerobic growth of *Enterobacteriaceae*, crotonobetaine could be used as an external electron acceptor during anaerobic respiration and that it plays the same role as compounds such as fumarate (Kröger, 1978; Cole *et al.*, 1985) or nitrate (Haddock and Jones, 1977). Other *Enterobacteriaceae* genera e.g. *Salmonella thyphimurium* and *Proteus vulgaris* are also not able to use L-carnitine as sole carbon and nitrogen source in aerobic conditions, but reduce L-carnitine into γ-butyrobetaine (*via* crotonobetaine) under anaerobic conditions (Jung *et al.*, 1993) in the presence of a carbon and nitrogen source (Fig. 2.11). Crotonobetaine plays the role of an external electron acceptor (Seim and Kleber, 1988). L-carnitine, crotonobetaine and acetyl-L-carnitine are stimulating the anaerobic growth of *Salmonella thyphimurium* in that their degradation product, γ-butyrobetaine, is directly responsible for it (Seim *et al.*, 1982c).

In *E. coli* 25922, *Proteus vulgaris* and *Proteus mirabilis,* L-carnitine degradation can occur either aerobically or anaerobically. However, the aerobic enzymatic activities are extremely low compared to their activity levels in obligatory aerobic *Proteus spp.* (Engemann and Kleber, 2001).

Crotonobetaine reductase and carnitine dehydratase (or hydrolase) are both localized in the cytosol (Jung *et al.*, 1987; Engemann and Kleber, 2001). Carnitine dehydratase activity has been detected during anaerobic growth and was shown to be reversible and inducible by L-carnitine or crotonobetaine (Table 2.3). This enzyme is not involved in the fatty acid metabolism of *E.coli*, does not require any energetic compound, is not stimulated by coenzyme A and is competitively inhibited by D(+)-carnitine and γ-butyrobetaine (Jung *et al.*, 1989).

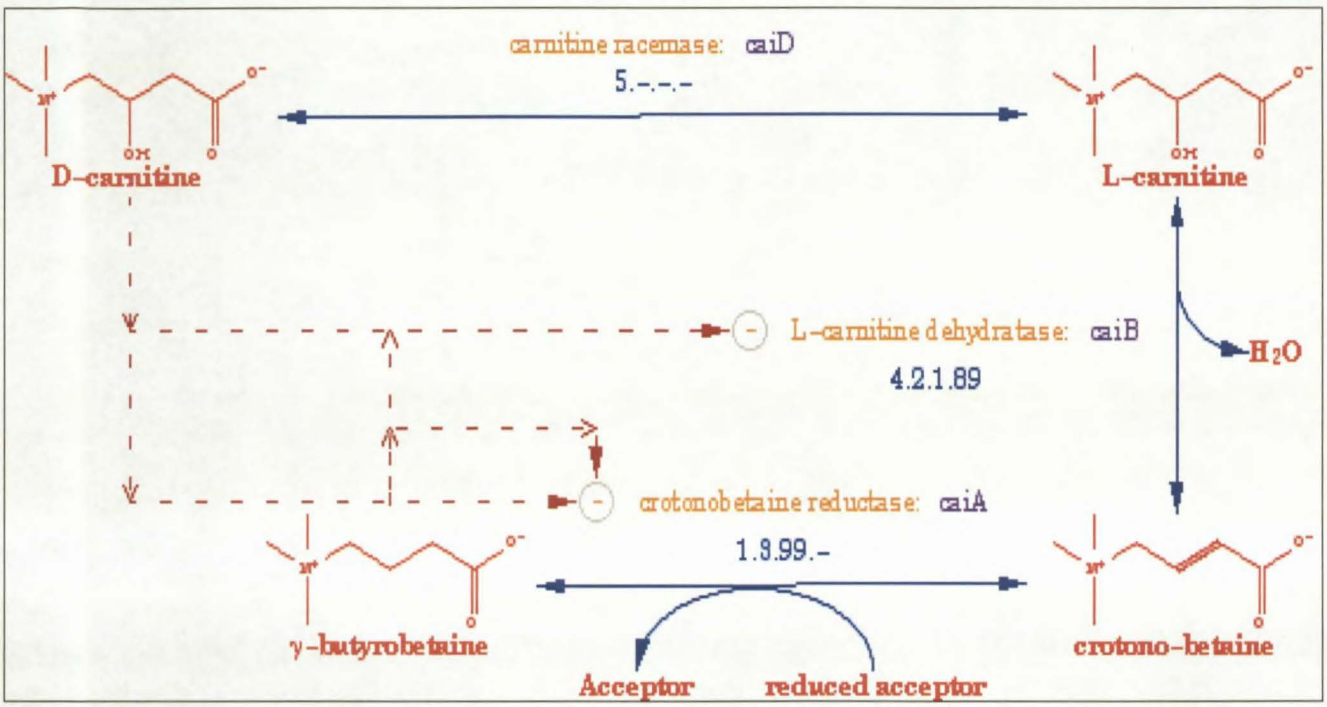

Figure 2.11 Carnitine metabolism in *E. coli*: L-carnitine is dehydrated into crotonobetaine, which is reduced in γ-butyrobetaine (Seim and Kleber 1988; Jung *et al.*, 1989, 1990; Jung and Kleber, 1991; Eicher *et al.*, 1994; Castellar *et al.*, 1998).

Bacteria that are able to reduce L-carnitine to γ-butyrobetaine, in the presence of other carbon and nitrogen sources, include *E. coli*, *Salmonella typhimutium*, *Proteus vulgaris*, *Proteus mirabilis* and *Citrobacter freundii*. During aerobiosis, L-carnitine is being degraded but crotonobetaine and γ-butyrobetaine are not detected (Elssner *et al.*, 1999).

2.3.3.2 Other roles of L-carnitine in bacteria

During osmotic stress (aero- or anaerobic) conditions, L-carnitine can act as an osmoprotectant similar to betaine or proline. In such cases, L-carnitine accumulates in the cytoplasm to increase the inner osmotic pressure in reaction to a high osmolarity level in the microenvironment to avoid dehydration (Jung *et al.*, 1990).

A four to six fold increase in cellular growth rate of *E. coli* O44K74 in a media containing 0.65 M NaCl was observed when the media was supplemented with L-carnitine at a concentration of 1mM. Interestingly, an increase in L-carnitine concentration to 10 mM did not have a significant additional effect, showing that only small quantities are required

(Jung *et al.*, 1990). Jung and associates demonstrated that the L-carnitine accumulation in the cell was proportional to the sodium chloride concentration. Moreover, comparing anaerobic and aerobic conditions, it appeared that L-carnitine accumulates in the cell only during stress conditions and that it was later catabolized to barely detectable levels (Jung *et al.*, 1990).

Table 2.3 Metabolism of L-carnitine after cultivation under anaerobic conditions with glycerol as carbon source and addition of different trimethylammonium compounds (Jung *et al.*, 1987).

Trimethylammonium compound (10mM)	**Specific activity of whole cells (carnitine metabolizing activity) (mol/h . mg protein)**
L-carnitine	2.29
D-carnitine	0
Crotonobetaine	2.18
γ-butyrobetaine	0
Choline	0
Glycine betaine	0

To restore turgor pressure, cells preferably accumulate energy-rich metabolites such as proline, glycine, betaine and carnitine rather than inorganic ions such as K^+ or Na^+ (Peluso *et al.*, 2000). This is also due to the fact that high intracellular salt concentrations can cause defects in other metabolic functions and disturb transmembrane potentials (Yancey *et al.*, 1982; Somero, 1986).

L-carnitine has already been shown to be a compatible solute for *Brevebacterium linens* (Jebbar *et al.*, 1998), *Lactobacillus plantarum, Bacillus subtilis* (Kets *et al.*, 1994), *Pseudomonas aeruginosa* (Lucchesi *et al.*, 1995) and *Listeria monocytogenes* (Smith, 1996). As for *Lactobacillus plantarum*, L-carnitine uptake can occur simultaneously with glycine and betaine even when NaCl concentration increases to 2,5M (Poolman and Glaasker, 1998). The protective role of L-carnitine is not restricted to turgor maintenance, but it can act as a cryoprotectant (Becker *et al.*, 2000) as well. In *Listeria monocytogenes*, two distinct responses to a cold shock have been demonstrated: (i) membrane fluidity adjustment through alteration of fatty acids composition and; (ii) accumulation of a compatible solute such as glycine, betaine, carnitine or proline, of which carnitine is the better cryoprotectant (Becker *et al.*, 2000).

In both cases, carnitine could play a role as a carrier during fatty acid changes undergone by adaptation to low temperature. In *Listeria monocytogenes*, the structure of the L-carnitine transporter is unknown, but it is likely to be ATP-dependent. In both osmotic and low-temperature stress, the general sigma factor σ^B could play a role as it is active after 20 min of stress and is stimulated by osmotic shock and by cold temperature shock (Becker *et al.*, 2000; Verheul *et al.*, 1997). The activity of σ^B is growth dependent and it could

modulate the collecting of L-carnitine during the logarithmic growth phase. In similar conditions, stationary phase cells were unable to grow (Becker *et al.*, 2000), suggesting a growth phase-dependent pathway for carnitine uptake during the stress response of *Listeria monocytogenes*.

The carnitine transporter of *Listeria monocytogenes* is ATP-dependent (Verheul *et al.*, 1995), and intragenous betaine and L-carnitine both inhibit the import. This transporter has a high affinity for carnitine, acetylcarnitine and γ-butyrobetaine, but an extremely low affinity for betaine and proline.

For the halophilic bacterium *Tertagenococcus halophila*, which requires 0.5 to 30 % of salt concentration to grow (with optimal growth at 10 %), a supplementation of 2 mM L-carnitine improves the growth in hyper- and hypo-osmotic medium (NaCl). *T. halophila* does not catabolize L-carnitine under aerobic conditions, and collects L-carnitine for osmoprotection. L-carnitine inhibits glutamate, proline and aspartate accumulation and would increase the intracellular potassium concentration at high salinity (Robert *et al.*, 2000).

It has been suggested that prokaryotic osmostressed cells also accumulate carnitine to stabilize membrane proteins (Scholte *et al.*, 1996; Peluso *et al.*, 2000), but further investigations have to be done on the eventual involvement of carnitine in protein folding, subunit aggregation and multiprotein complex stability, the preservation of enzyme stability, and the protection of enzymes against denaturation by solutes like urea (Peluso *et al.*, 2000).

2.3.3.3 Genes involved in carnitine metabolism in *E. coli*

*E. c*oli is the most frequently used bacterium in microbiological and genetic research. It has been extensively studied, and is well characterized and its genome entirely sequenced. As a consequence, much of the information available on carnitine metabolism in bacteria is concerning *E. coli*. The *E. coli* transport system for L-carnitine is not specific to L-carnitine but generally transports trimethylamine carboxylic-like molecules, and D-carnitine, crotonobetaine, γ-butyrobetaine and glycine betaine can also bind to the substrate site. L-carnitine transport is temperature dependant and strongly reduced at low temperatures.

To date, three carnitine uptake system have been found in *E. coli* (ProP, ProU, CaiT). During osmotic stress, L-carnitine accumulation in the *E. coli* cytoplasm is ensured by ProP, a proline /betaine transporter (Table 2.4), and by the proteins encoded by the ProU operon (Table 2.5), as well as more specifically by CaiT (Table 2.6).

2.3.3.3.1 ProU and ProP

The ProU and ProP transport systems were initially described for proline and betaine uptake. ProP and ProU are not specific for carnitine, and are respectively proline/betaine and glycine-betaine/proline transporters. Both are stimulated at high osmolarity levels and ProP presents a lower affinity for carnitine (Verheul *et al.*, 1998).

Proteins homologous to ProP and ProU have also been found in *Bacillus subtilis* (Kappes *et al.*, 1996; Kempf and Bremer, 1995), and proteins homologous to ProP in *Erwinia chrysanthemi* (Gouesbet *et al.*, 1996). For *Lactobacillus plantarum*, the transport of quaternary ammonium compounds is depending on a unique transport system (QacT). This transport shows an affinity (Km) for glycine betaine and carnitine fifty times higher than for proline (Glaasker *et al.*, 1998).

Table 2.4 Characteristics of ProP

Gene	Protein	Protein length (aa)	Mol. Weight	Function	Subcellular location	Similarity
ProP	Proline/ betaine trans-porter.	500	54845	Strech-inactivated proline/betaine transporter. ProP is both an osmo-sensor and osmoregulator, which is available to participate early in the bacterial osmoregulatory response. Mediates the active accumulation of solutes such as proline, glycine betaine, stachydrine, pipecolic acid, ectoine and taurine.	Integral membrane protein. Inner membrane.	Belongs to the sugar transporte r family.

Table 2.5 Characteristics of ProX/U.

Gene	Protein	Protein length (aa)	Molecular weight (Daltons)	Function	Subcellular location	Similarity
Pro X/U	Glycine betaine-binding periplas mic protein (precurs or).	330	36022	Member of a multicomponent binding-protein-dependent transport system (the ProU transporter) which serves as the glycine-betaine/L-proline transporter.	Periplasmic.	

Another operon, *caiTABCDE*, is encoding a specific transporter of carnitine and is encoding genes involved in carnitine metabolism.

2.3.3.3.2 *Cai TABCDE* operon

The L-carnitine catabolic pathway in *E. coli* is encoded by the *caiTABCDE* operon, which comprises in its 5' region the *fix ABCX* operon (Buchet *et al.*, 1999) (Fig. 2.12). *Cai TABCDE* and *fix ABCX* operons are co-expressed anaerobically and have to be induced by L-carnitine or crotonobetaine (Eichler *et al.*, 1994; Eichler *et al.*,1995; Buchet *et al.*, 1998). Genes from the *fix ABCX* are involved in electron transfer to crotonobetaine reductase (CaiA) during crotonobetaine reduction to γ-butyrobetaine (Eichler *et al.*, 1996).

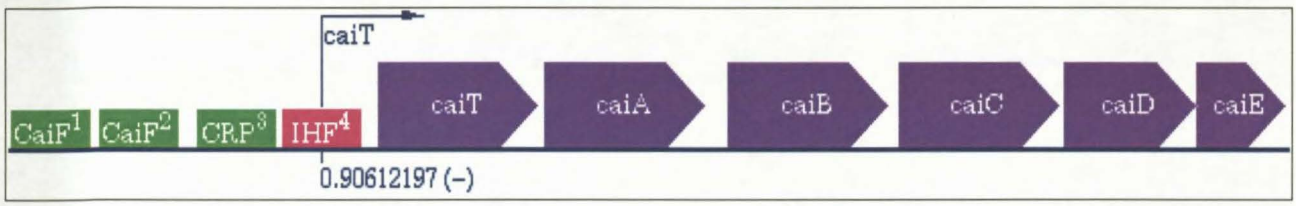

Figure 2.12 Schematic representation of the operon caiTABCDE (from http://ecocyc.org, 2002).

These operons are both repressed by molecular oxygene, glucose and nitrate and both require the transcription factor for aerobic regulation (FNR) and the cAMP receptor protein CRP responsible for the activation of catabolic operon. Both are also repressed by nucleotide associated proteins (H-NS). The two operons are likely to be dependent on the transcription of CRP and CaiF for their activation. A common region encoding three CRP binding sites, between the transcription start site of the promoter of these two operons (Buchet *et al.*, 1998) has been identified. The highest CRP affinity site is responsible for *cai* activation, and the two others are postulated as activators of the *fix* operon (Eichler *et al.*, 1996).

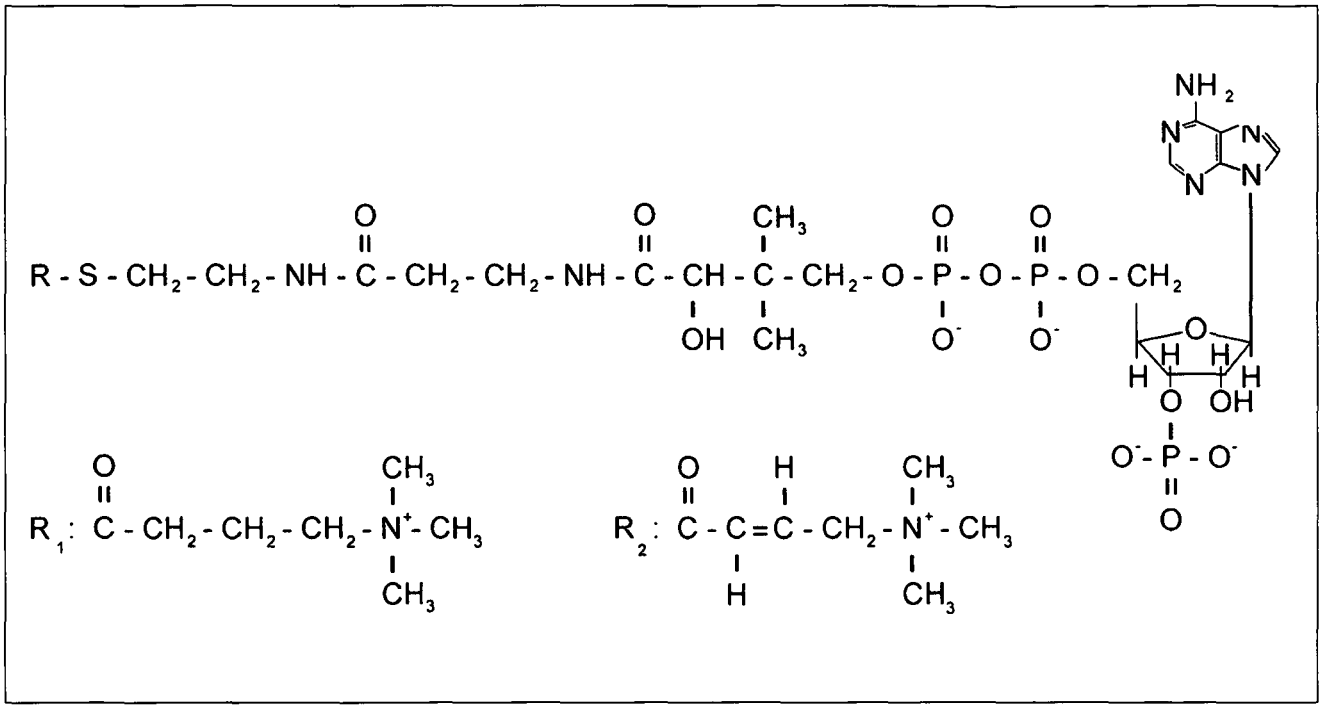

Figure 2.13 Structure of γ-butyrobetainyl-CoA (1) and crotonobetainyl-CoA (2) (from Dykes and Moorhead, 2000).

The role of the different proteins encoded by the operon is indicated in Table 2.6. CaiA is an oxido-reductase, and *caiA* and *caiB* together are encoding the crotonobetaine reductase, comprising two dimers of CaiB associated with a tetramer of CaiA (Dykes and Moorhead, 2000).
The enzymatic reactions that are catalyzed by crotonobetaine reductase are

Reduced acceptor + crotonobetaine $\rightleftharpoons$ acceptor + γ-butyrobetaine
Reduced acceptor + crotonobetainyl-CoA $\rightleftharpoons$ acceptor + γ-butyrobetainyl-CoA

Under anaerobic conditions, γ-butyrobetainyl-CoA synthesis is induced by L-carnitine or crotonobetaine, and repressed by oxygen. As oxygen is also repressing crotonobetaine reductase activity, it is highly probable that mechanisms involved in biosynthesis of this cofactor and those inducing carnitine metabolic enzymes would be the same and would be subjected to an identical genetic control (Buchet *et al.*, 1999).

A probable crotonobetaine/carnitine CoA ligase is encoded by *caiC*, and the presence of both crotonobetainyl-CoA and γ-butyrobetainyl-CoA (Fig. 2.13) could be explained by a transformation of γ-butyrobetainyl-CoA to crotonobetainyl-CoA (Dykes and Moorhead, 2000).

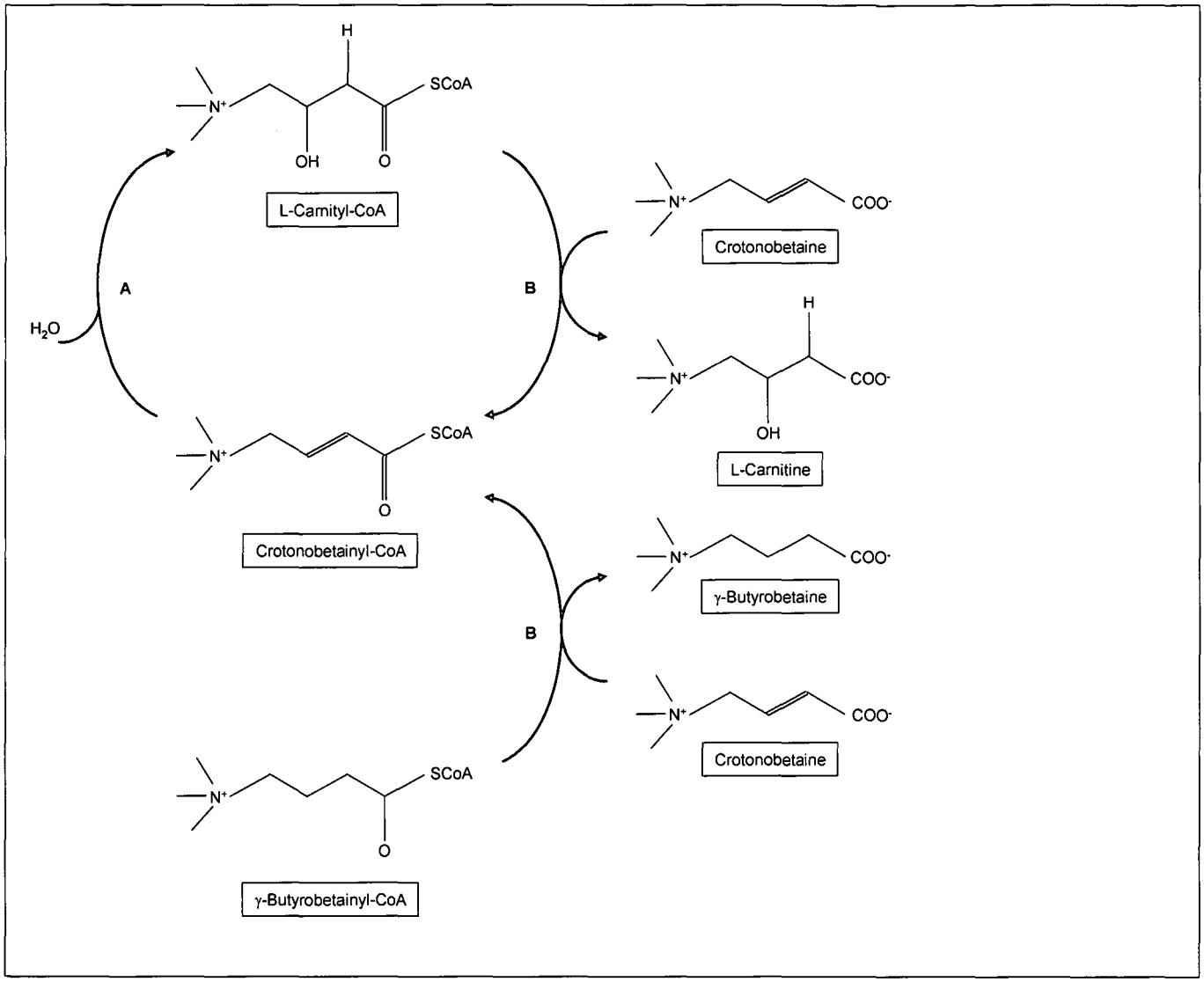

Figure 2.14 Mechanism proposed by Elssner *et al.* (2000) for the transformation of crotonobetaine to L-carnitine catalyzed by either L-carnitine dehydratase or an enzyme system consisting of an enoyl-CoA hydratase (A) and a CoA transferase (B).

CaiB is encoding the L-carnitine dehydratase, comprising two dimers of CaiB. CaiB is located in the cytoplasm (Fig. 2.14). Enzymatic reactions catalyzed by L-carnitine dehydratase are:

L-carnitinyl-CoA $\rightleftharpoons$ H_2O + crotonobetainyl-CoA
L-carnitine $\rightleftharpoons$ H_2O + crotonobetaine

L-carnitine dehydratase catalyses the first step of L-carnitine metabolism. It has also been isolated from *Pseudomonas putidia* (62kDa, two identical subunits), and from *Xanthomonas translucens* (74 kDa). The trimethylammonium and carboxyl groups are binding to the substrate site (Goulas, 1988). *CaiB* gene expression requires the regulation protein CRF for its activation and, is repressed by H-NS proteins (Eichler *et al.*, 1994).
CaiC is a crotonobetaine/carnitine-CoA ligase. The enzymatic reaction catalyzed by this ligase are the following:

CoA-SH + D-carnitin ⇌ D-carnitinyl-CoA
CoA-SH +crotonobetaine ⇌ crotonobetainyl-CoA
CoA-SH + L-carnitine ⇌ L-carnitinyl-CoA

The *caiD* gene codes for a carnitine racemase catalysing two reactions in carnitine metabolism:

D-carnitinyl-CoA ⇌ L-carnitinyl-CoA
D-carnitine ⇌ L-carnitine

According to Elssner *et al.* (2000), it is highly probable that the physiological substrate is CoA linked.

The *caiE* gene encodes a putative enzyme involved in the synthesis or in the activation of an unknown cofactor. This cofactor is required for the activities of both carnitine dehydratase and carnitine racemase. Elssner and co-workers (2000) showed that a CaiE overproduction stimulates the activities of carnitine racemase and dehydratase. CaiE could be the enoyl-CoA hydratase required for the transformation of crotonobetainyl-CoA to L-carnitine CoA.

CaiF, encoded by the *caiF* gene, is a 15 kDa protein responsible for the induction of cai and fix operons (Eichler *et al.*, 1996), it also regulates their anaerobic expression (Buchet *et al.*, 1998). The transcription factor for anaerobic growth (FNR) is positively controlling *caiF* transcription (Eichler *et al.*, 1996; Elssner *et al.*, 2000).

Buchet and co-workers (1999) suggested that the *fix* operon transcription activation requires a complex formation of two CRP activators and CaiF.

CaiT belongs to the BCCT family (the betaine, carnitine choline transporters) and is located in the inner membrane. It is a putative proton-motive-force driven uptake system for carnitine.

Table 2.6 Proteins encoded by the *caiABCDEFT* operon.

Gene name	Protein name	Protein length (AA)	Molecular weight (DA)	Function	Subcellular location	Similarity
CaiA	Probable carnitine operon oxidoreduc-tase CAI A.	380	42558	Involved in the reduction of crotonobetainyl-CoA into γ-butyrobetainyl-CoA. Pathway: carnitine metabolism.	-	Belongs to the acyl-CoA dehydro-genases family.
CaiB	L-carnitine dehydrata-se.	405	45126	Catalyzes the aerobic dehydration of L-carnitine to crotonobetaine. Catalytic activity: L-carnitine = 4-(trimethylammonio) -but-2-enoate +H_2O. A cofactor is necessary for its activity. This cofactor is found only in cells grown under anaerobic conditions and in presence of carnitine (probably crotonobetainyl-CoA). Pathway: carnitine metabolism, conversion of carnitine to γ-butyrobetaine. Subunit: homodimer. Induction by L-carnitine or crotonobetaine.	Cytoplasmic.	To bile acid-inducible OPERON protein F (BAI F) from Eubacte-rium sp. and to E.coli YFDE.
CaiC	Probable crotonobe-taine/carni-tine-CoA ligase.	522	59089	Could catalyses the transfer of CoA to crotonobetaine or carnitine. Pathway: carnitine metabolism.	Integral membrane protein. Inner membrane (potential).	Belongs to the AMP-binding enzyme family.
CaiD	Carnitine racemase.	297	32311	Catalytic activity: D-carnitine to L-carnitine. Pathway: carnitine metabolism.	-	Belongs to the enoyl-CoA hydratase/i somera-se family.
CaiE	Carnitine operon protein CaiE.	196	21244	Pathway: carnitine metabolism.	-	Belongs to the CYSE/LAC A/ LPXA/NOD L family of acetyl transfera-ses. Compo-sed of multiple reapeats of [LIV]-G-X.

Table 2.6 (cont.)

Gene name	Protein name	Protein length (AA)	Molecular weight (DA)	Function	Subcellular location	Similarity
CaiF	Transcrip-tional activatory protein CaiF.	131	15436	Potential transcriptional activator of carnitine metabolism.	-	-
CaiT	Probable carnitine transporter.	504	56587	Probable proton-motive force driven uptake of carnitine and analogous. Pathway of carnitine metabolism.	Integral membrane protein. Inner membrane.	Belongs to the BCCT family of transpor-ters.

A study of *caiTABCDE* and *fixABCX* operons of *E.coli* showed several common aspects. Both are essential for L-carnitine metabolism during anaerobic growth, activated by CRP and CaiF, co-expressed anaerobically in presence of L-carnitine, influenced by glucose and nitrate, positively regulated by CPR and FNR, and negatively regulated by H-NS proteins. The area of 289 bp in the intergenic region is regulatory and contains three potential CRP binding sites (Table 2.7; Buchet *et al.*, 1998).

Table 2.7 Proteins encoded by the *Fix ABCX* operon

Gene	Protein	Protein length (AA)	Molecular weight (DA)	Function	Sub-cellular location	Similarity
FIX A	Fix A protein.	256	27143	May play a role in a redox process involving CAI A. Pathway: carnitine metabolism. Subunit: Fix A and Fix B from a heterodimer (probable).	-	Belongs to the ETF β-bubunit/ Fix A family.
FIX B	Fix B protein.	313	33513	May play a role in a redox process involving CAI A. Pathway: carnitine metabolism. Subunit: Fix A and Fix B from a heterodimer (probable).	-	Belongs to the ETF α-bubunit/ Fix B family.
FIX C	Fix C protein.	428	45702	Probably accepts electrons from Fix A/Fix B and reduces a quinone. Cofactor: FAD (potential).	-	Belongs to the ETF-QO/Fix C family.
FIX X	Ferredo xin like protein	95	10479	Could be a 3FE-4S cluster containing protein. Probably participates in a redox process with Fix A, Fix B and Fix C.	-	To various bacterial ferredoxins, strongest to Fix X from nitrogen fixing bacteria.

2.3.3.4 Bioreactors for carnitine synthesis

From the understanding of carnitine metabolism in the *Enterobacteriaceae* emerged the development of bioreactors for L-carnitine synthesis as an alternative to chemical methods. The main problem with these chemical processes is the racemic production of carnitine, and according to the side effects of D-carnitine — including its inhibiting effect on human pyruvate dehydrogenase — a carnitine mix cannot be used in therapy (De Simone and Famulario, 1997; Merck Index 2001). Due to the cost of chiral separation, L-carnitine was only available at high prices. The development of the use for L-carnitine in different medical therapeutic practices are increasing the need for L-carnitine.

Using the reversible and inducible properties of these enzymes, and the fact that L-carnitine metabolism is mainly anaerobic, it is possible to switch carnitine metabolism to an aerobic L-carnitine anabolism.

Once Jung *et al.* (1989) had demonstrated the existence of a carnitine racemase, Castellar and coworkers (1998) used the capacities of *E. coli* to convert anaerobically D-carnitine to L-carnitine. This method led to an L-carnitine production of 0.55 g/l.h with *E .coli* O44K74 (Castellar *et al.*, 1998). D-carnitine as a waste product becomes a cheap substrate.
Although oxygen is known to be an inhibitor of enzymes involved in L-carnitine catabolism such as the crotonobetaine reductase (Seim and Kleber 1988; Obòn *et al.*, 1999), some crotonobetaine was found in the reaction mix, in a concentration superior than the reverse reaction of L-carnitine dehydratase is able to produce. A new enzyme transforming D-carnitine to crotonobetaine was proposed and named D-carnitine dehydratase (from all these information a model for carnitine metabolism in *E. coli* has been deducted (Fig. 2.15).

During this biotransformation process, it has been noticed that fumarate increased the level of metabolic activities and the production of L-carnitine.
Fumarate is a terminal acceptor of phosphorylative electron transport (Kröger, 1978), and as a result the reaction does not tend towards the production of γ-butyrobetaine. Moreover, the energy gained by the fumarate reduction is available for D-carnitine uptake (Jung *et al.*, 1990).

Another kind of bioreactor using the same type of *E. coli* strain but crotonobetaine as substrate has been presented (Obòn *et al.*, 1999). This model of reactor was suitable for industrial L-carnitine production as the biocatalytic capacity led to a synthesis of 6.2 g of L-carnitine/l.h.

2.4 CONCLUSION

Carnitine clearly is an important organic molecule with many different roles in different organisms. Most of the known physiological roles and the underlying genetic and

metabolic functions have been described in some detail in this review. While it is therefore clear that a significant amount of information regarding the main metabolic functions of carnitine has been generated, this review also clearly highlights many open questions regarding the roles of carnitine particularly in eukaryotic cells. The research described in the following chapters of this thesis was designed to contribute new knowledge regarding such additional metabolic roles of this compound.

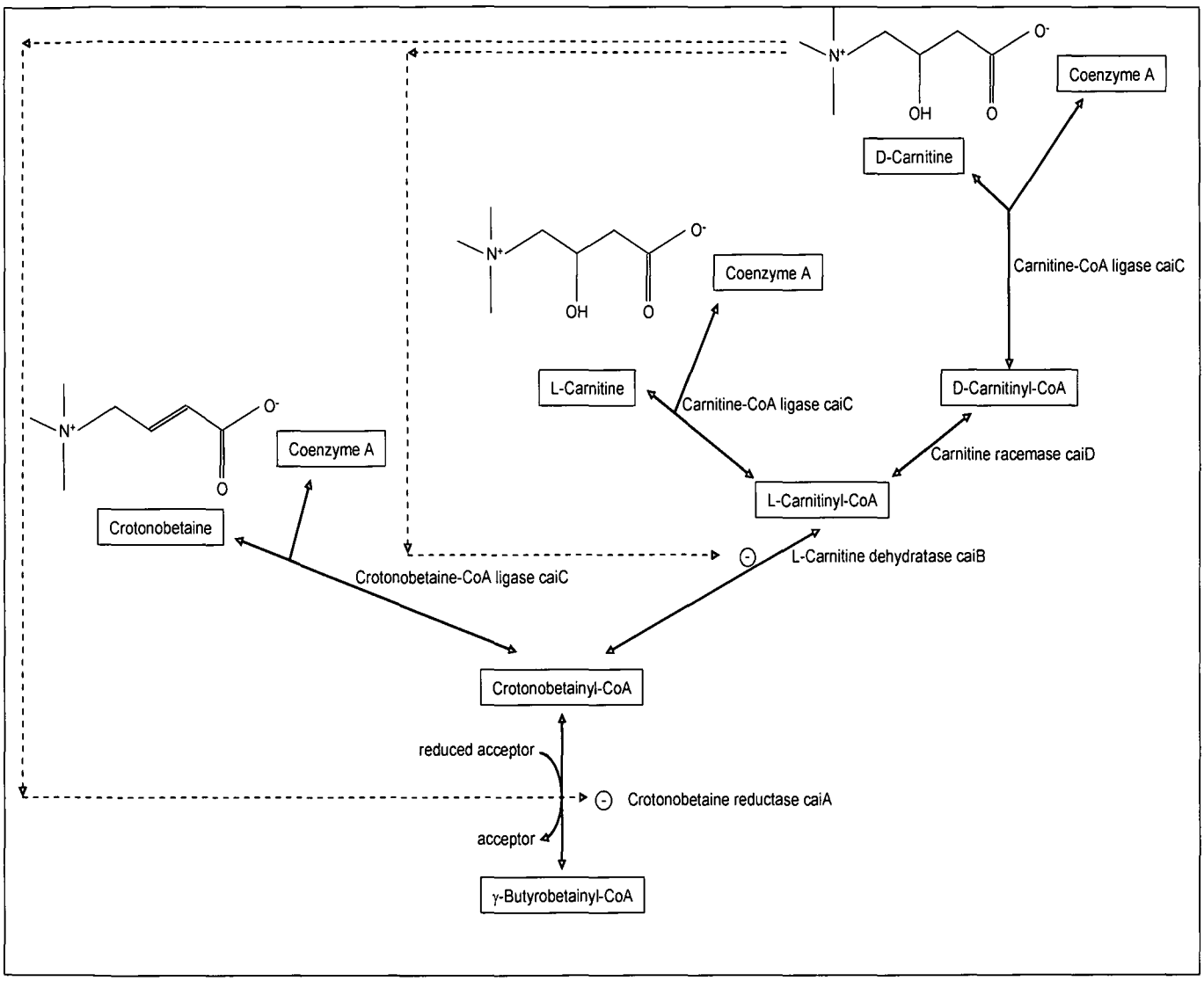

Figure 2.15 Carnitine pathways in *E. coli* (from ecocyc.org, 2002).

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CHAPTER 3

RESEARCH RESULTS

INVESTIGATING THE EFFECTS OF L-CARNITINE ON YEAST PHYSIOLOGY

3. RESEARCH RESULTS

3.1 INTRODUCTION

The main focus of this M.Sc. study was to investigate the influence of carnitine on the physiology of yeast cells. More particularly, the research focused on the role of carnitine in stress resistance. The study furthermore attempted to link some newly discovered phenotypes to specific genes. The results obtained in this investigation are presented in the following chapter.

In an associated project, conducted in collaboration with J.H. Swiegers and S. Kroppenstedt, a HPLC-Electrospray Mass Spectrometry method to measure carnitine and derivates (acetyl-carnitine) levels during our experiments was developed. These results are presented as an article in the appendix.

3.2 RESEARCH RESULTS

A new role for carnitine in yeasts

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3.2.1 ABSTRACT

L-Carnitine is a quaternary ammonium compound whose main role in the metabolism of eukaryotic cells is the shuttling of activated acyl residues. Recent epidemiological data on the clinical application of L-carnitine have raised questions about potential additional roles for this compound to explain the multiple benefits observed when administrated as a complementary medicine. In order to identify possible unknown physiological roles of carnitine, the effects of this compound on various aspects of the cellular physiology of yeast, and in particular of *Saccharomyces cerevisiae*, including on general and invasive growth as well as osmotic and heat stress resistance, were investigated. To assess whether the observed effects were specific for some strains of *S. cerevisiae* or an indication of a more general effect of L-carnitine in yeast and therefore perhaps in other eukaryotic organisms, several strains and two additional yeast species were also investigated for at least some of the phenotypes. In this report, we show that exogenously added L-carnitine clearly improved survival and stimulated growth under hyperosmotic and thermal stress conditions. Using a genetic screen, the *PHO87* gene which has previously been identified as encoding a low-affinity inorganic phosphate carrier was shown to contribute to this protective effect. Cells over-expressing the gene showed improved survival to hyperosmotic stress and higher levels of intracellular carnitine than the corresponding wild type strain. The data suggest that L-carnitine can act as a compatible solute to counter hyperosmotic stress. However, this explanation can not account for the improved survival of cells exposed to thermal stress.

3.2.2 INTRODUCTION

L-Carnitine is a small water soluble compound (Mw=162) present in most organisms, including vertebrates, invertebrates, plants, fungi and bacteria. In eukaryotes, carnitine is involved in the metabolism of fatty acids and more particularly in the transfer of activated

acyl residues across intracellular membranes (Bieber, 1988). In humans, L-carnitine ensures the transfer of activated fatty acids from the cytosol to the mitochondrion, balances acetyl-CoA levels between the mitochondrial matrix and the cytosol, and enhances glucose oxidation by stimulating pyruvate dehydrogenase. In yeast, it is known to shuttle activated acetyl-residues from the peroxisomes or the cytoplasm to the mitochondria for further catabolization (Bieber, 1988; Osmundsen *et al.*, 1991; Reddy and Mannaerts, 1994).

In humans, between 54% to 87% of carnitine, depending on the type of diet adopted, is provided through dietary intake, while the remainder is synthesized *de novo* from lysine and methionine, mainly in the liver (Rebouche *et al.*, 1984; Rebouche, 1992). Although mammals are not able to degrade carnitine, microorganism of the intestinal flora can catabolize the molecule (Rebouche *et al.*, 1984; Seim *et al.*, 1985). These studies show that carnitine is not involved in fatty acid metabolism, but can be used as a carbon or nitrogen source. Furthermore, in some bacteria L-carnitine accumulates in the cytoplasm during hyperosmotic stress, and is used as a compatible solute (Kunau *et al.*, 1995). Other studies show that when switching from aerobic to anaerobic conditions, *Escherichia coli* is able to shift from L-carnitine catabolism to carnitine synthesis using either D-carnitine, crotonobetaine or γ-butyrobetainyl-CoA as precursors (Jung *et al.*, 1990)(see annexe 2).

During the past decade, the extension of carnitine supplementation for patients suffering of various diseases, including AIDS, Reye's syndrome (encephalopathy), chronic fatigue syndrome, Parkinson's and Alzheimer's diseases as well as patients after surgery indicated that carnitine can contribute to significantly improve the overall health status of many patients. These data also suggested that carnitine may be involved in other metabolic or physiological mechanisms in eukaryotic cells. As direct studies on higher eukaryotes are complex and frequently problematic, yeasts, and especially *Saccharomyces cerevisiae*, can be used as an eukaryotic model system.

In *S. cerevisiae*, as in all eukaryotic organisms, carnitine has been shown to be instrumental in the shuttling of activated acetyl-residues between cellular compartments (van Roermund *et al.*, 1999). This yeast, however, is unable to synthesize carnitine *de novo* (van Roermund *et al.*, 1999; Swiegers *et al.*, 2001). According to the carbon source that is provided for growth, acetyl-CoA is formed in different cellular compartments. In the case of growth on fatty acids as sole carbon source, acetyl-CoA is formed in the peroxisomes through β-oxidation (Kunau *et al.*, 1995). In these conditions, the activated acetyl has to be transferred to the mitochondria for further catabolism. However, organellar membranes are impermeable to acetyl-CoA. For this reason, the activated acetyl residues are transferred to a molecule of carnitine in a reaction catalyzed by carnitine acetyl transferases (CATs). Three genes encoding such enzymes, *CAT2*, *YAT1* and *YAT2*, have been identified in *S. cerevisiae*, but the specific roles of two of those genes, *YAT1* and

YAT2, remain to be clarified. Acetyl-carnitine can be transported between compartments through the action of acetylcarnitine/carnitine translocases (van Roermund *et al.*, 1999). After transfer of acetylcarnitine to the mitochondria, the acetyl residues can be transferred back to a molecule of free CoA-SH, allowing further catabolism and associated energy generation through the tricarboxylic acid cycle (TCA) and oxidative phosphorylation. This metabolic process is referred to as the carnitine shuttle, and all three *CAT*-encoding genes have been shown to be essential for the shuttle to be functional (van Roermund *et al.*, 1998, 1999; Swiegers *et al.*, 2001). However, the carnitine shuttle is not essential for the growth or survival of *S. cerevisiae* in any of the conditions investigated thus far, including growth on fatty acids as sole carbon source. Another metabolic pathway, the partially peroxisomal glyoxylate cycle, can indeed serve as a metabolic bypass to the carnitine shuttle (van Roermund *et al.*, 1999). In wild type cells of *S. cerevisiae*, deletion of carnitine shuttle genes therefore does not lead to any detectable phenotypes.

In this study, the effects of extracellular carnitine on the physiology of several strains of the yeast *S. cerevisiae* are investigated. To ensure that any observed effect of carnitine is of a general nature and not specific to one type of yeast, two other yeast species, *Yarrowia lipolytica* and *Pichia pastoris*, are also investigated for some of the relevant phenotypes. These two species were chosen because, contrarily to *S. cerevisiae*, both were shown to be able to neo-synthesize carnitine (unpublished data). Several physiological tests were conducted in the presence of various concentrations of carnitine. The data show that carnitine significantly improves survival of yeast cells subjected to hyperosmotic and hyperthermal stress. In the case of hyperosmotic stress, the data indicate that carnitine may act as a compatible solute. The data furthermore indicate that the *PHO87* gene, previously identified as an inorganic phosphate carrier (Bun-ya *et al.*, 1996), contributes significantly to the observed phenotypes.

3.2.3 RESULTS

3.2.3.1 CARNITINE CAN NOT BE USED AS CARBON OR NITROGEN SOURCE BY *S. CEREVISIAE*

Before investigating the effects of carnitine on the physiology of *S. cerevisiae*, the various strains used in this study were assessed for their ability to use carnitine as either a nitrogen or carbon source. For this purpose, yeast strains *S. cerevisiae* FY23, PSY142, ISP15 and W303 were spotted onto YNB minimal media without either carbon or nitrogen source and with or without 20 g/l or 2 g/l of L-carnitine, respectively. No difference in growth was observed between plates that contained or did not contain carnitine for any of

the four strains investigated, confirming that *S. cerevisiae* can not use carnitine as a nutrient (data not shown).

3.2.3.2 CARNITINE DOES NOT HAVE ANY EFFECT ON INVASIVE OR FILAMENTOUS GROWTH

When *S. cerevisiae* is faced with a limitation of carbon or nitrogen sources, the yeast may under certain conditions switch to an elongated pseudohyphal growth form (Gimero et *al.*, 1992). Invasive growth assays and observations of pseudohyphal cells using an optical microscope were conducted with plates of *Yarrowia lipolytica*, *Pichia pastoris* and the four *S. cerevisiae* strains as described in the Material and Methods section. The data indicate that the presence at various concentrations or the absence of carnitine in the growth substrate had no detectable impact on the ability of cells to invade agar. Furthermore, no differences in pseudohyphal development and the morphological appearance of cells invading the agar could be observed in the presence or absence of carnitine (Fig 1).

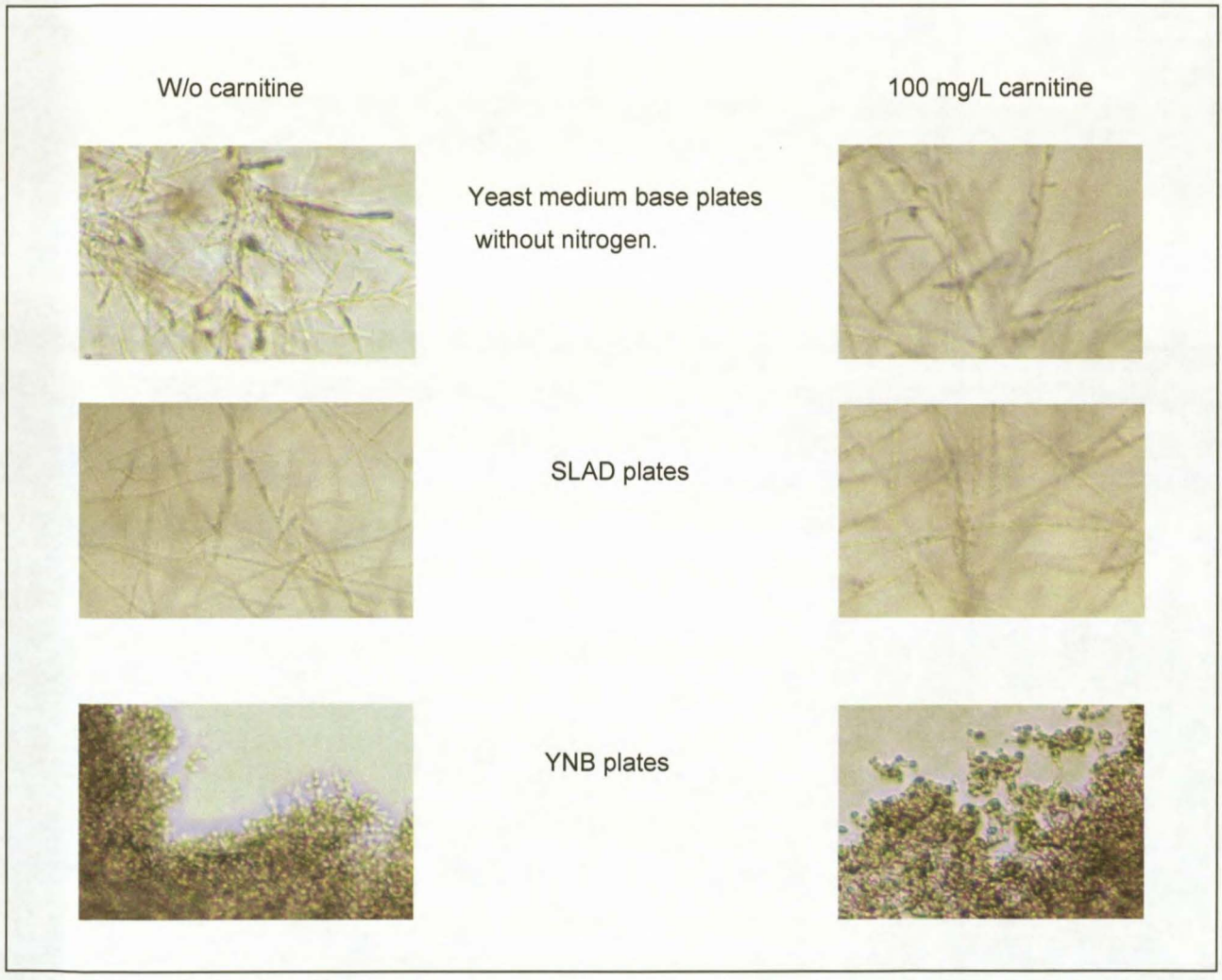


Figure 1. Pseudohyphal assays of *Yarrowia lipolytia*. Cells grown in YNB were spotted as described onto SLAD medium plates and controls plates with or without L-carnitine.

3.2.3.3 CARNITINE IMPROVES YEAST HYPEROSMOTIC STRESS SURVIVAL

To assess whether L-carnitine could affect yeast cells exposed to hyperosmotic stress, exponentially growing cultures of the various *S. cerevisiae* strains and *Y. lipolytica* were spotted onto plates containing a range of concentrations of NaCl or sorbitol, as well as different concentrations of carnitine (1, 10 and 100 mg/l) as described in the Materials and Methods section. The data showed that the intrinsic sensitivity toward osmotic stress was different for the two yeast species, with *Y. lipolytica* being significantly more resistant than *S. cerevisiae*. However, both species showed marked improvement in survival in the presence of high levels of carnitine in the medium. Indeed, Figures 2 and 3 clearly show that the addition of 100 mg/l of L-carnitine to the media significantly improved the survival of both *S. cerevisiae* and *Y. lipolytica* to hyperosmotic stress in the presence of NaCl. All *S. cerevisiae* strains showed similar behaviours, although the intrinsic stress resistance of each strain was slightly different.

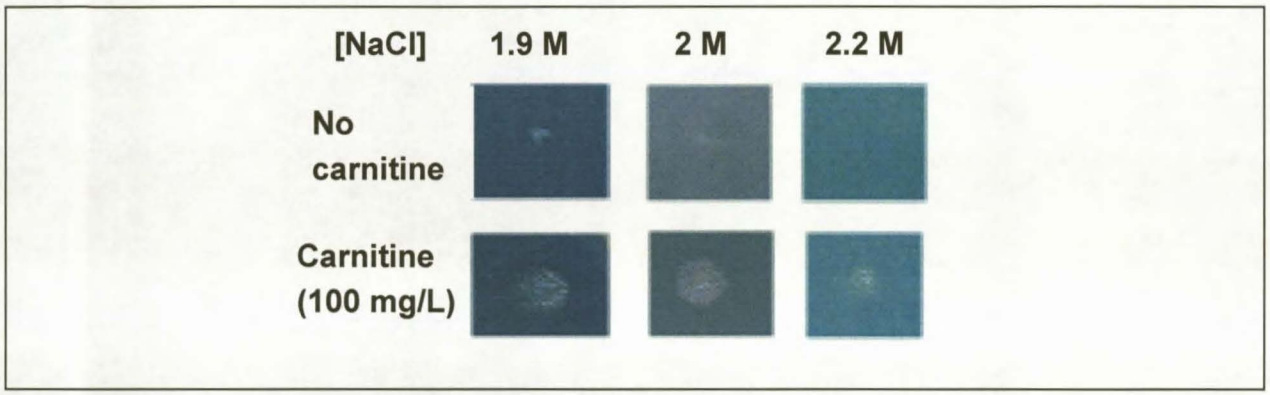


Figure 2. Survival of *S. cerevisiae* strain W303 to hyperosmotic shock. A total of 5 µl (10^5 cells) of W303 were spotted onto YNB plates containing the indicated concentrations of NaCl and with or without 100 mg/l of L-carnitine.

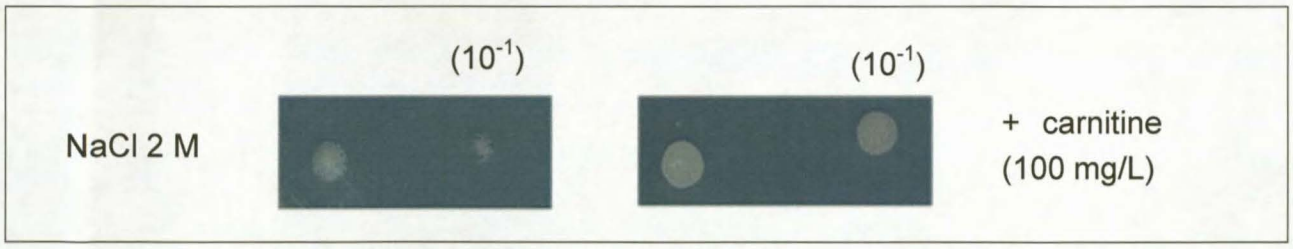
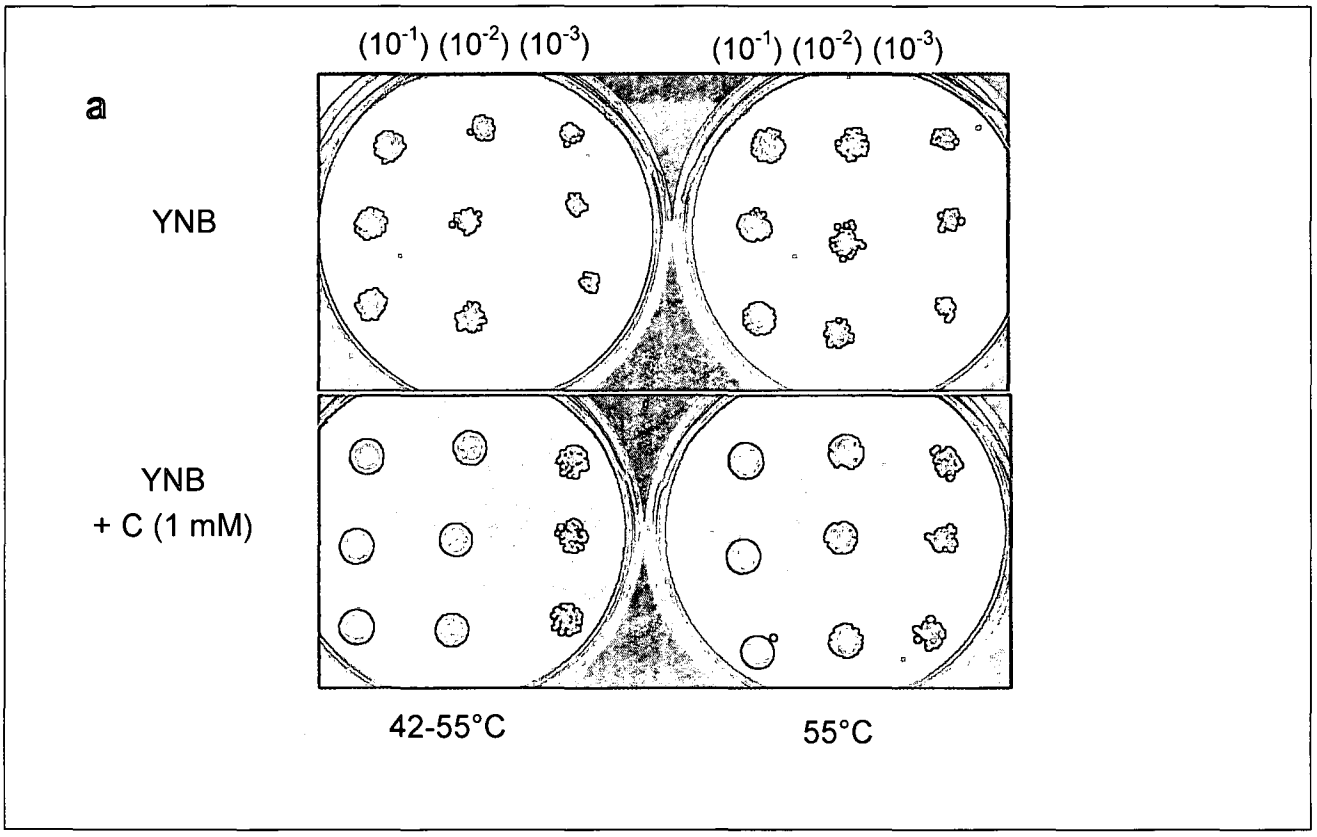


Figure 3. Survival of *Y. lipolytica* to hyperosmotic shock. Cells of *Y. lipolytica* were spotted as described (dilutions to 10^{-1} in water). YNB contained 2M of sodium chloride and was supplemented with L-carnitine at 100 mg/L.

When sorbitol was used as an osmotic stress inducer similar data were obtained (data not shown), although survival rates at similar concentrations of osmolyte were generally higher than with NaCl. This can probably be attributed to the fact that NaCl also generates salt toxicity in addition to osmotic stress. The effect of carnitine was strongest at the highest concentration (100 mg/l), and weakest at lowest concentration, indicating that the compound could act as a compatible solute (data not shown).

To further investigate optimal stress protection concentrations of carnitine, strain *S. cerevisiae* W303 was exposed to hyperosmotic stress using YNB plates containing 1.8M NaCl and carnitine concentrations of 100 mg/L; 150 mg/L, 200 mg/L; 400 mg/L; 600 mg/L; 800 mg/L, and 1 g/L (data not shown). No further improvement was observed above 200mg/L. Further tests indicated that the same applied to a concentration of 162 mg/L (1 mM).



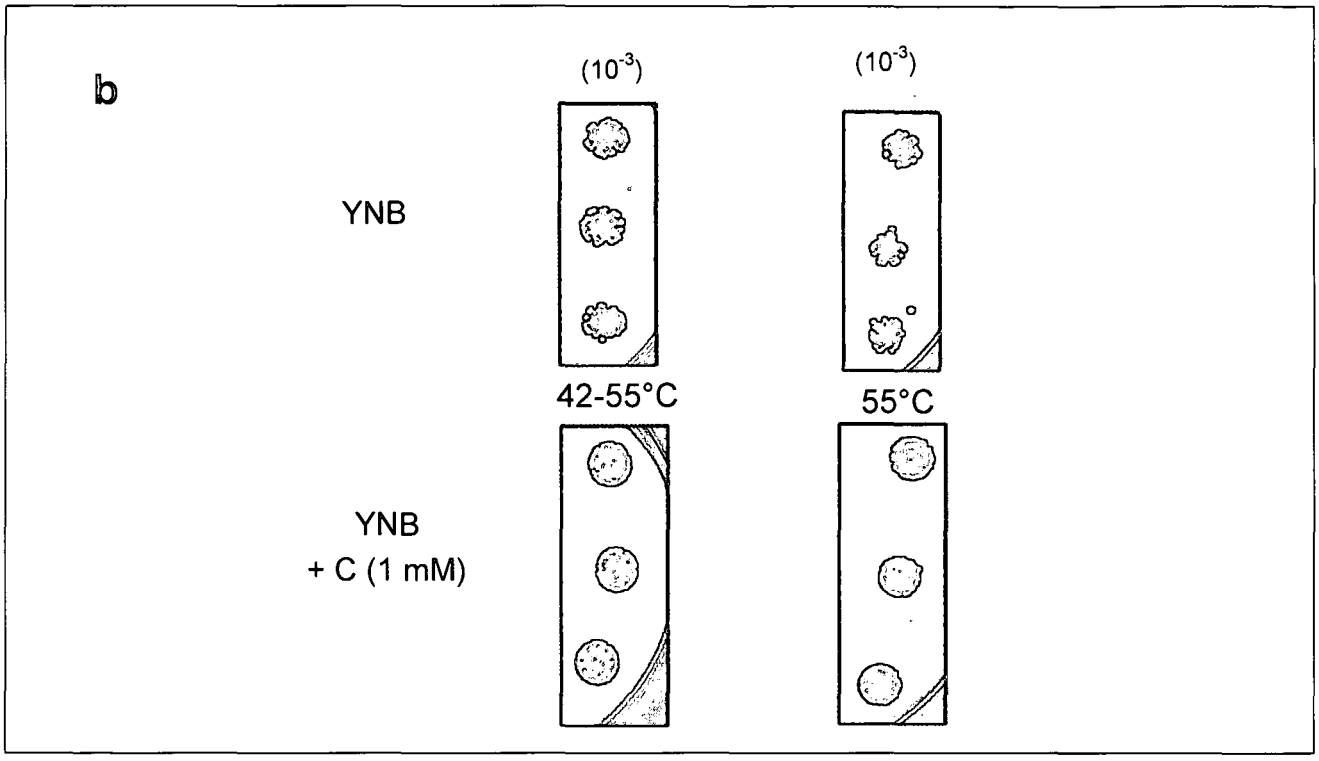


Figure 4a and b. Heat shock resistance of *S. cerevsiae* strains. Cultures of *S. cerevisiae* strains L-5366 (Fig. 4a) and FY23 (Fig 4b) were grown in YNB to mid-log phase ($O.D._{600}$ = 1) and 2.5 µl of the culture were spotted in three different dilutions (10^{-1}, 10^{-2} and 10^{-3}, corresponding to 10^4, 10^3 and 10^2 cells per spot, respectively) onto YNB plates with or without 1 mM of carnitine. Plates were then incubated at high temperatures with (42°C-55°C) or without pre-adaptation period (55°C), after which they were incubated at 30°C for two days and photographed.

The effect of carnitine on hyperosmotic stress survival and growth were also assessed in liquid media. Figure 3 shows the growth pattern of the FY23 strain in YNB containing 1.8 M of NaCl in the presence or absence of 1mM of L-carnitine. The data show that the presence of carnitine in these conditions resulted in a significantly reduced lag-phase and a significantly increased final optical density, confirming the protective effect of carnitine.

3.2.3.4 CATS AND ACETYLCARNITINE ARE NOT INVOLVED IN THE PROTECTIVE ACTIVITY

To investigate whether the *S. cerevisiae* genes that encode the enzymes responsible for the synthesis of acetylcarnitine and that are essential for the carnitine shuttle are required for the observed phenotypes, a mutant strain deleted for all three identified carnitine acetyl transferases, FY23Δ*yat1*Δ*yat2*Δ*cat2* (FY23ΔCATS), was analysed in similar conditions as the FY23 wild-type when growing in YNB supplemented with 1.8 M NaCl (Figure 5). The triple mutant showed exactly the same growth behaviour as the wild-type, with carnitine supplementation providing the same degree of protection to both strains. Plate assays using this strain also confirmed that hyperosmotic shock tolerance was unaffected by the absence of these genes (data not shown). Addition of 1mM of acetylcarnitine (AC) to the

media did not result in any protective effect. These data clearly suggest that neither the carnitine shuttle nor acetylcarnitine are required for the osmoprotective activity of carnitine.

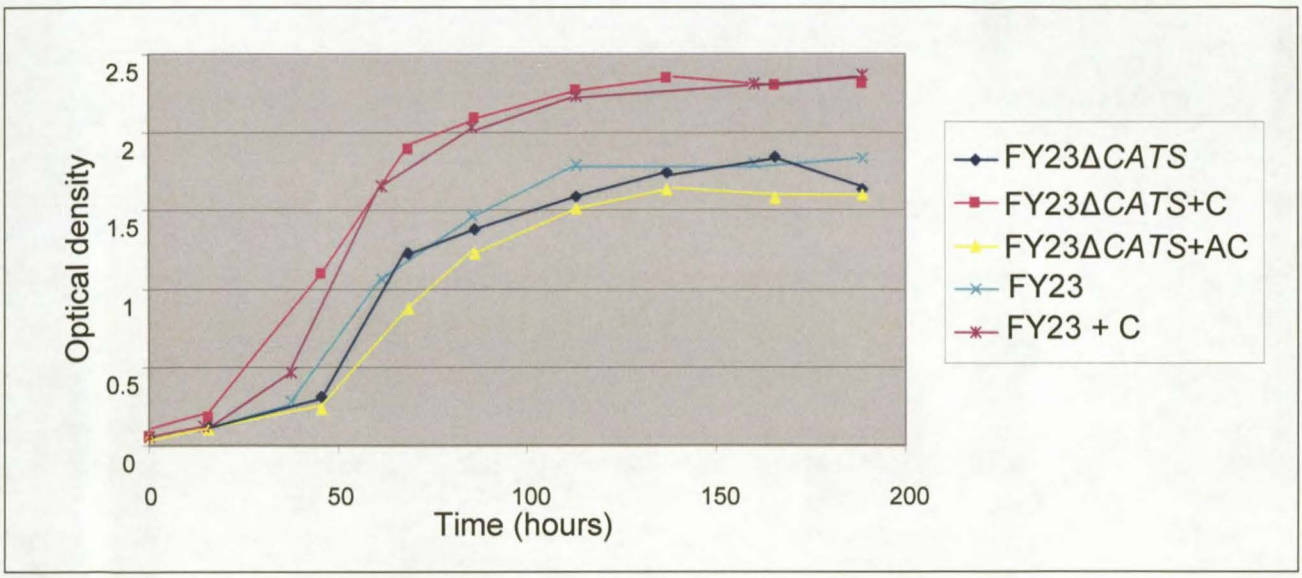


Figure 5. Growth of *S. cerevisiae* FY23 wild type and of the isogenic triple carnitine acetyl transferase mutant FYΔCATS (FY23Δ*yat1*Δ*yat2*Δ*cat2*) during growth on 1.8 M NaCl supplemented or not with carnitine or acetylcarnitine (1 mM). The presence of carnitine is indicated as +C, while +AC indicates acetylcarnitine.

3.2.3.5 CARNITINE IMPROVES YEASTS HEAT SHOCK SURVIVAL

To assess if carnitine could be involved in other protective mechanisms, thermal stress experiments were conducted. These heat shock experiments, carried out as described in the Materials and Methods section, indicated that survival of all tested yeast species and of the different strains of *S. cerevisae* was improved in the presence of 1 mM of carnitine (Figures 4a and b).

Control plates grown at 30°C did not result in any growth improvement when supplemented with carnitine (data not shown). The improvement in survival due to the presence of carnitine was more marked on YNB when compared to rich YPD media. Also, the protective effect was reduced for preconditioned cells.

3.2.3.6 *AGP2* IS NOT REQUIRED FOR OSMO-TOLERANCE

Agp2 is the only known transporter responsible for the uptake of carnitine. To verify the role of this protein in osmotic stress protection, *S. cerevisiae* FY23 wild type and the isogenic BY4742Δ*agp2* mutant strain were assessed for their response to hyperosmotic stress. Indeed, if carnitine acts as a compatible solute, transport of the compound into the cell is essential for the protective effect. As expected, the survival of the *AGP2* deleted strain was clearly reduced compared to the *AGP2* WT strain. Transport by Agp2p

therefore clearly contributes to the protective effect. However, and as can be seen in Fig. 6, carnitine still exerts a significant protective effect in this strain. This may indicate the existence of another protein able to transport carnitine into the cell.

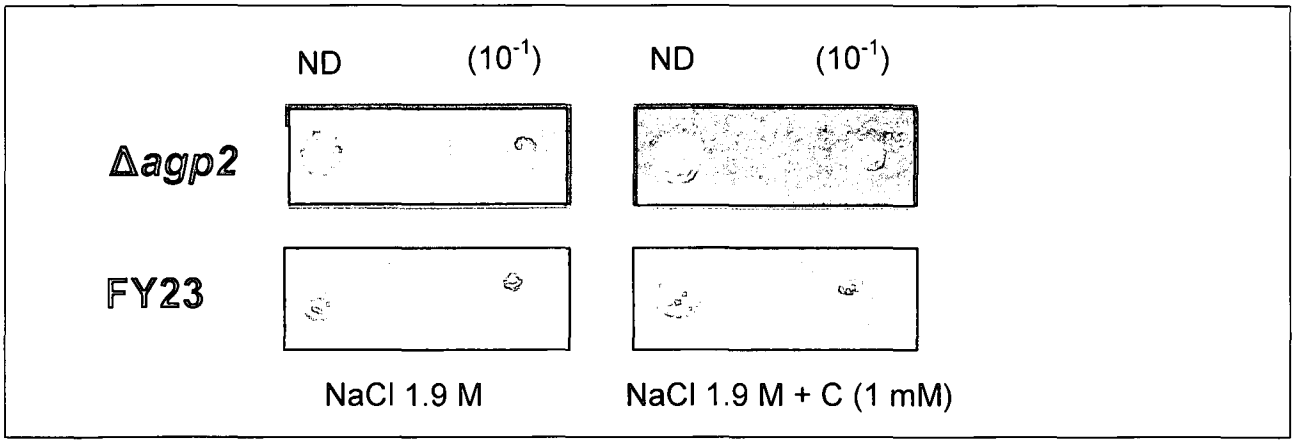


Figure 6. *AGP2* is not essential for carnitine-dependent osmo protection. *S. cerevisiae* BY4742Δ*agp2* mutant cells, and FY23 wild-type were spotted in concentrations of 10^4 (ND) and 10^3 (10^{-1}) cells per spot on YNB containing 1.9 M NaCl in the presence or absence of 1 mM carnitine.

The data suggest that carnitine can be taken up by the cell in the absence of Agp2p, and that other transporters of carnitine may exist in *S. cerevisiae*.

3.2.3.7 *PHO87* CONTRIBUTES TO THE PROTECTIVE EFFECT OF CARNITINE

To identify genes that may be involved in the newly identified stress resistance phenotypes, we analysed the proteome of *S. cerevisiae* to identify proteins that show homologies with those that had been identified in carnitine related pathways in *E. coli* (see also annexe 2). Table 1 shows the result of the BLAST alignments. In addition to those genes, we also included the gene *PHO87* into this analysis. This gene had previously been identified in our laboratory during the cloning of suppressors of *CIT2* deletion phenotypes. *CIT2* encodes the peroxisomal citrate synthase, the first enzyme of the glyoxylate cycle. These deletion mutants are viable, but are dependent on carnitine for growth on fatty acids as carbon source because of a dysfunctional glyoxylate cycle (Swiegers *et al.*, 2001). The growth defect of *CIT2* deletion strains was partially suppressed by the presence of *PHO87* on a multiple copy plasmid (unpublished data).

The corresponding BY4742 mutants (Table 1) were tested for heir ability to withstand hyperosmotic stress (1.8 M NaCl) in the presence or absence of 1 mM of carnitine. Tests were also conducted in both aerobic and anaerobic conditions, since the corresponding pathway in *E. coli* is repressed in the presence of oxygen. No differences were observed for the two conditions. The results of these assays are indicated in Table 2. Three of the deleted strains showed no noticeable improvement in the presence of carnitine, indicating a possible role of these genes in carnitine-dependent stress protection. They were

BY4742Δ*pho87*, BY4742Δ*ypr153w* and BY4742Δ*fat2*. BY4742Δ*ypr153w* and BY4742Δ*fat2* presented the same phenotypes as shown for BY4742Δ*pho87* in Figure 7. The selected genes *PHO87*, *YPR153w* and *FAT2* need to be analyzed further before drawing any conclusion.

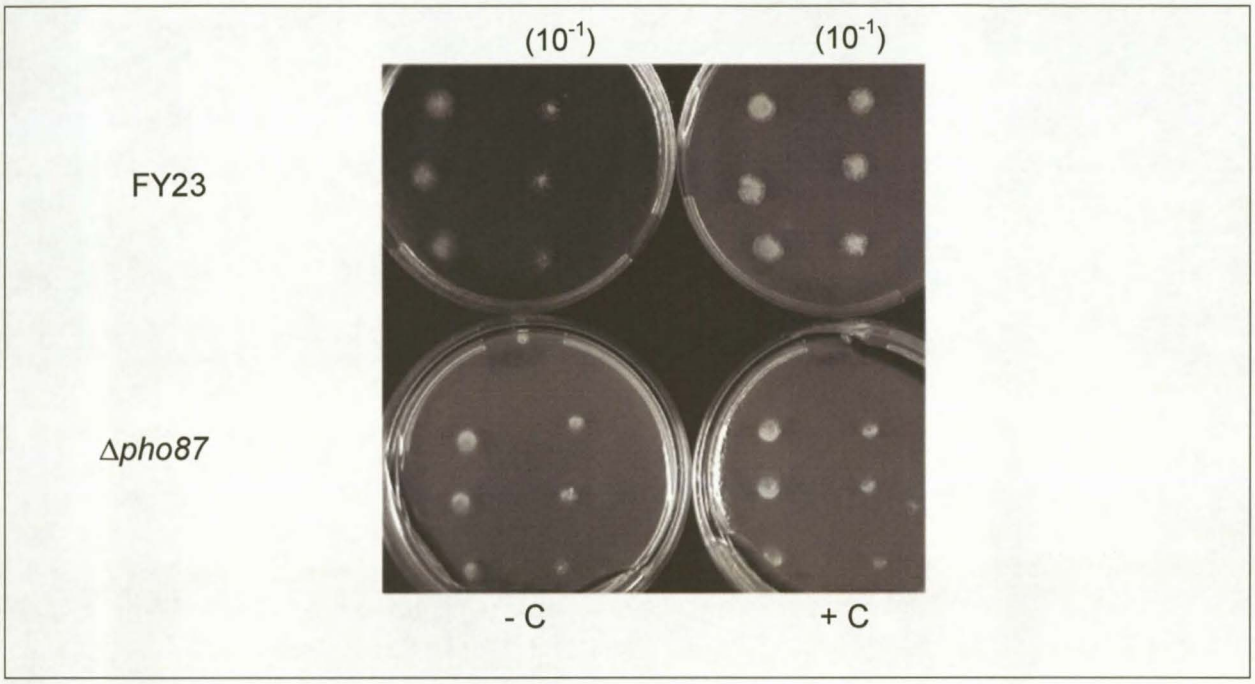


Figure 7. Osmotic stress resistance of *S. cerevisiae* FY23 wild-type and BY4742Δ*pho87*. Cells were spotted on YNB containing 1.8 M of NaCl. The precultures were grown in YNB with or without carnitine to mid-log phase ($O.D._{600}$ of 1) and 2.5 μl were spotted onto the plates. The plates on the right hand side contain 1 mM of carnitine.

Table 1. Results of *E. coli* protein alignment with *S. cerevisiae* proteome and the corresponding proteins with their functions.

***E. coli* protein**	**Function in *E. coli***	***S. cerevisiae* homologues**
ProP	Osmoregularory carnitine proline betaine transporter (stretch activated)	*PHO84* *JEN1*
ProU	Transport system which serves for the glycine-betaine and L-proline	*MPM1*
CAIC	Crotonobetaine/carnitine CoA ligase	*FAT1* *FAT2*
CAID	Carnitine racemase	*YDRO36C*
CAIT	Probable carnitine transporter	*YPR153W* *YNR002C*

Table 2. Mutant strain tested for carnitine-dependent improvement of stress survival.

BY4742 mutants tested	**Results** Improvement in presence of carnitine	**Previously identified role of gene**
Δ*pho84*	Like WT	High-affinity inorganic phosphate (Pi) transporter and low-affinity manganese transporter
Δ*pho87*	**NO IMPROVEMENT**	Low-affinity inorganic phosphate (Pi) transporter, involved in activation of PHO pathway
Δ*mpm1*	LIKE WT	Mitochondrial membrane protein of unknown function
Δ*jen1*	LIKE WT	Lactate transporter, required for uptake of lactate and pyruvate
Δ*ypr153w*	**NO IMPROVEMENT**	**Hypothetical protein**
Δ*ynr002c*	LIKE WT	Putative transmembrane protein
Δ*fat1*	LIKE WT	Fatty acid transporter and very long-chain fatty acyl-CoA synthetase
Δ*fat2*	**NO IMPROVEMENT**	**Peroxisomal AMP-binding protein**
Δ*ydr036c*	LIKE WT	Protein of unconfirmed function, plays an indirect role in endocytic membrane trafficking

3.2.3.8 *PHO87* OVEREXPRESSION INCREASES HYPEROSMOTIC AND HYPERTHERMAL SHOCK RESISTANCE

YEp (Yeast Episomal plasmids) are multiple copy plasmids and are commonly used to increase expression levels of specific genes. The *PHO87* open reading frame was cloned into YEp352 under the control of the strong, constitutive *PKG1* promoter. When transformed into *S. cerevisiae*, such a plasmid ensures very high expression of the corresponding mRNA. YEp-*PHO87* was transformed into FY23, and the phenotype of the strain was compared to the one of the same strain transformed with YEp352 without the *PHO87* ORF. The data show a significant growth improvement in the presence of 1 mM of carnitine for the *PHO87* transformed strain compared to the control strain under hyperosmotic stress conditions (Figure 8).

To verify if Pho87p could also play a role in heat stress survival the intrinsic thermo tolerance and induced thermo tolerance of strains FY23, FY23-YEp-*PHO87* and BY4742Δ*pho87* were tested in the same conditions as described previously. In these

conditions, FY23-YEp-*PHO87* showed higher survival than the wild type and the Δ*pho87* mutant (data not shown).

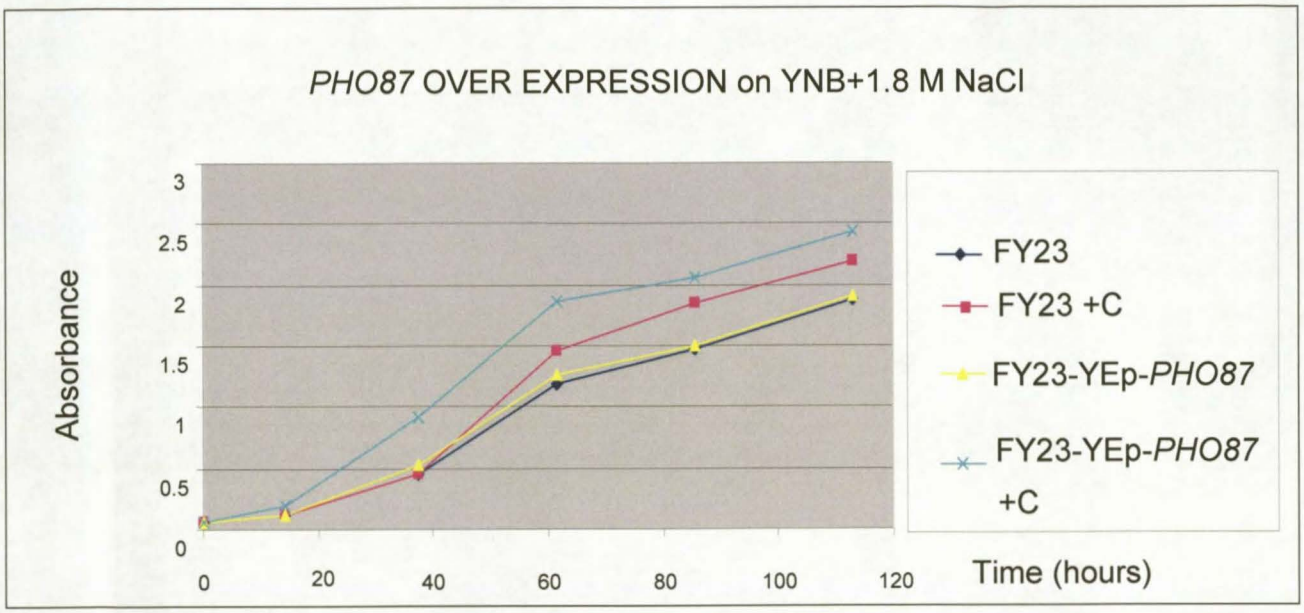


Figure 8. Growth of *S. cerevisiae* FY23-YEp-*Pho87* and FY23-YEp on YNB media containing 1.8 M NaCl and supplemented or not with carnitine (1 mM).

3.2.3.9 INCREASED EXPRESSION OF *PHO87* INCREASES CELLULAR CARNITINE LEVELS

To verify whether increased expression of *PHO87* affected carnitine concentrations in yeast cells, strains FY23-YEp, FY23-YEp-*PHO87* and BY4742Δ*pho87* were grown in YNB containing 1 mM of carnitine. After centrifugation and washing, the amount of carnitine in the cells was determined by ES-MS/MS. The data show that cells transformed with the *PHO87* over-expression construct contained higher total carnitine (carnitine and acetylcarnitine) levels than the wild type in the same conditions (Figure 9). Furthermore, the strain deleted for *PHO87* showed very low levels of carnitine, less than a third of the amount found in the FY23-YEp-PHO87.

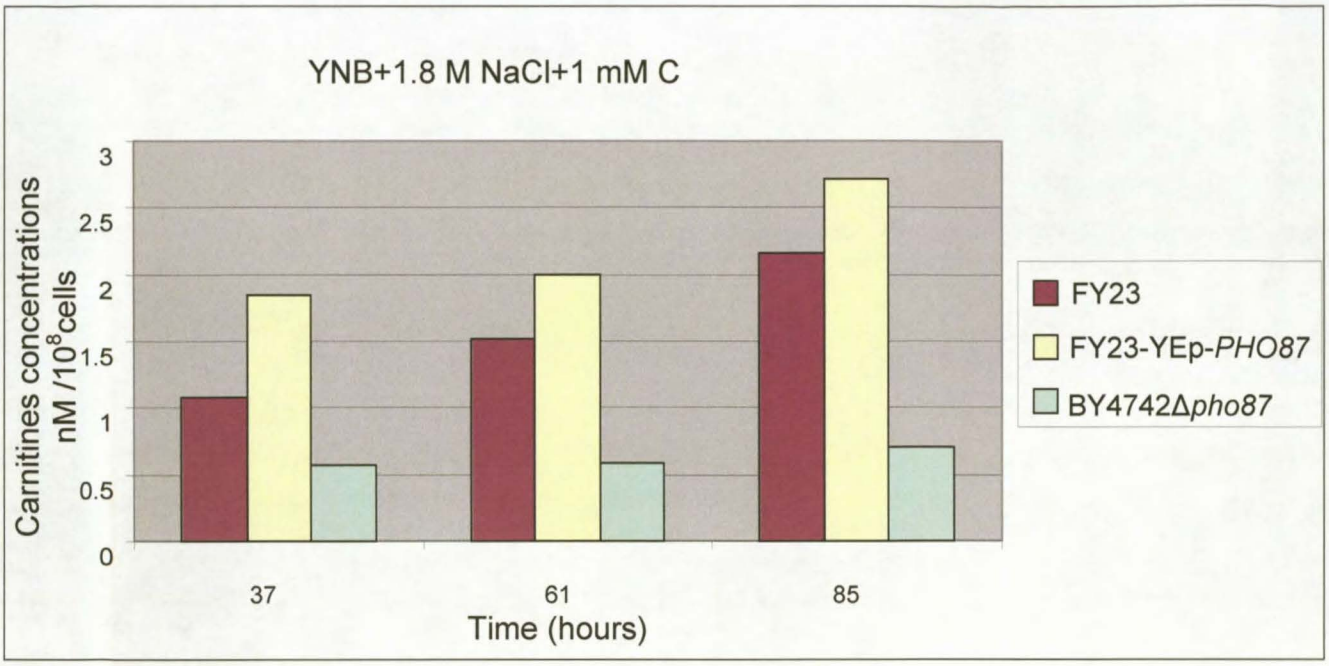


Figure 9. Carnitine content of *S. cerevisiae* FY23, FY23-YEp-*PHO87* and BY4742Δ*pho87*. Cells were grown in YNB media containing 1.8 M NaCl and 1 mM of carnitine. Carnitine was measured at three different stages of the growth phase after 37 hours (mid-log phase) 61 hours (entry of stationary phase) and 85 hours (stationary phase). Each measurement was made in triplicate, and the variation between triplicate measurements was less than 5% in all cases.

3.2.3.10 D-CARNITINE DOES NOT IMPROVE STRESS RESISTANCE

Replacing L-carnitine by its isomer D-carnitine in the same set of aerobic and anaerobic stress conditions showed that D-carnitine did not have any effect on the growth of FY23 wild type or on the BY4742Δ*pho87* and FY23-YEp-*PHO87*. No improvement was observed for osmotic stress or heat shock resistance. This experiment also suggests that *S. cerevisiae* does not possess a carnitine racemase.

3.2.3.11 YEAST APPEAR NOT TO HAVE OTHER PATHWAYS FOR CARNITINE

In order to investigate whether there might be other metabolic pathways using carnitine as a substrate in *S. cerevisiae*, a paper chromatography of aerobic and anaerobic hyperosmotic YNB cultures samples supplemented or not with carnitine were analysed by paper chromatography as described in the Material and Methods section (Figure 10).

Carnitine and acetylcarnitine were detected in the cells grown in the presence of carnitine but no other migrating dot lower than carnitine or above acetylcarnitine were detected under the conditions used. Carnitine therefore does not appear to be metabolised to any other compound.

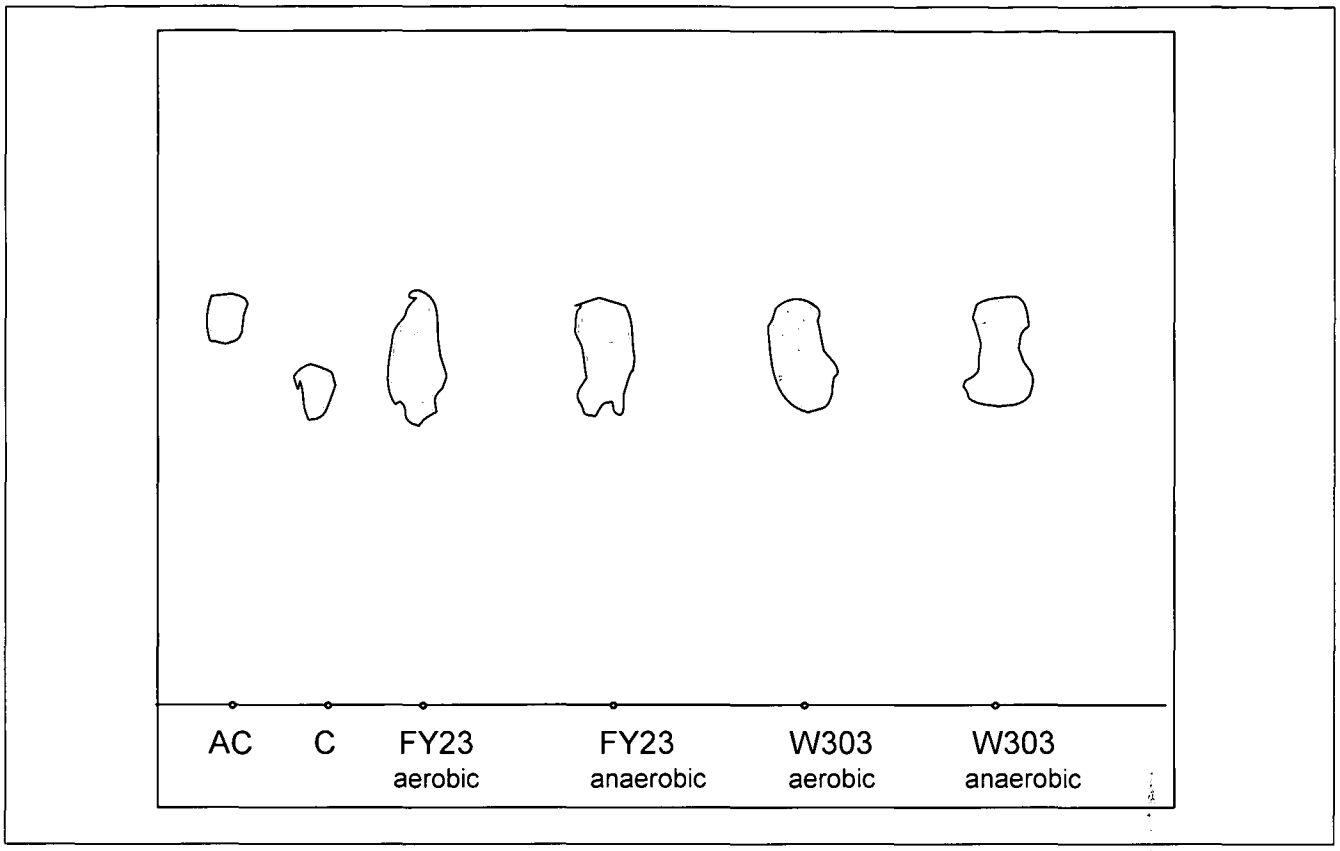


Figure 10. Schematisation of bioautogram of cell extracts. *S. cerevisiae* FY23 and W303 grown aerobically and anaerobically in YNB media containing 1.8 M NaCl and supplemented with carnitine (1 M).

3.2.4 EXPERIMENTAL PROCEDURE

3.2.4.1 YEAST STRAINS AND GROWTH CONDITIONS

The yeast species and strains used in this study are listed in Table 3. Yeast strains were grown at 30°C in standard Yeast Peptone Dextrose (YPD) containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose or in minimal Yeast Nitrogen Base (YNB) medium, containing 0.67% yeast nitrogen base without amino acids, 2% (w/v) glucose and supplemented with the required amino acids (Difco). Corresponding solid media contained 2% agar (Difco). Low-ammonium (SLAD) medium is similar to YNB, but containing only 50µM of ammonium sulphate as sole nitrogen source, and was prepared according to Lorenz and Heitman (1997). Carnitine and NaCl were added as described for each experiment. All yeast species and strains were assayed in triplicate, unless differently specified.

Table 3. Yeast species and strains.

Species and strains	Genotype	Source or reference
Yarrowia lipolytica W29	Wild-type	This laboratory
Pichia pastoris	Wild-type	This laboratory
Saccharomyces cerevisiae:		
FY23	*MATa leu2 trp1 ura3*	Winston *et al.*, 1995
FY23Δ*yat1*Δ*yat2*Δ*cat2*	*MATa* Δ*yat1*::*TRP1* Δ*yat2*::*LEU2* Δ*cat2*::*URA3*	S. Kroppenstedt
PSY142	*MATα leu2 lys2*	Kispal *et al.*, 1988
ISP15	*MATa his3 leu2 thr1 trp1 ura3 STA2*	This laboratory
W303	*MATa leu2 trp1 ura3*	This laboratory
L-5366	*MATa ura3*	This laboratory
FY10	*MATa leu2 ura3*	Winston *et al.*, 1995
BY4742	*MATα leu2 lys2 his3 ura3*	EUROSCARF
BY4742Δ*pho84*	*MATα leu2 lys2 his3 ura3* Δ*pho84*::*kanMX4*	EUROSCARF
BY4742Δ*pho87*	*MATα leu2 lys2 his3 ura3* Δ*pho87*::*kanMX4*	EUROSCARF
BY4742Δ*mpm1*	*MATα leu2 lys2 his3 ura3* Δ*mpm1*::*kanMX4*	EUROSCARF
BY4742Δ*jen1*	*MATα leu2 lys2 his3 ura3* Δ*jen1*::*kanMX4*	EUROSCARF
BY4742Δ*ypr153w*	*MATα leu2 lys2 his3 ura3* Δ *ypr153w*::*kanMX4*	EUROSCARF
BY4742Δ*ynr002c*	*MATα leu2 lys2 his3 ura3* Δ *ynr002c*::*kanMX4*	EUROSCARF
BY4742Δ*fat1*	*MATα leu2 lys2 his3 ura3* Δ*fat1*::*kanMX4*	EUROSCARF
BY4742Δ*fat2*	*MATα leu2 lys2 his3 ura3* Δ*fat2*::*kanMX4*	EUROSCARF
BY4742Δ*ydr036c*	*MATα leu2 lys2 his3 ura3* Δ*ydr036c*::*kanMX4*	EUROSCARF
BY4742Δ*agp2*	*MATα leu2 lys2 his3 ura3* Δ*agp2*::*kanMX4*	EUROSCARF

To determine whether carnitine could serve as a carbon and/or nitrogen source, *S. cerevisiae* strains FY23 and PSY142 were grown on media in which the carbon or nitrogen source was substituted with carnitine. For the carbon source test, strains were grown in liquid media containing 1.7 g/L YNB and 20 g/L glucose or 20 g/L of carnitine. For

the nitrogen source experiment, strains were grown in basic liquid media 1.7g/L YNB w/o ammonium sulphate and 20 g/L of glucose to which was added 5 g/L ammonium sulphate, 2 g/L glutamine or 2.2 g/L carnitine.

3.2.4.2 PLASMIDS AND YEAST TRANSFORMATION

The *PHO87* gene was amplified by means of the polymerase chain reaction (PCR) technique, with genomic DNA from the S288c genetic background serving as template and primers F-5'-CGAGAATTCATGAGATTCTCACACTTTCTC-3' and R-5'-GCTCTCGA GTTAAGTGCTACCTTTTAAGAC-3'. The unique *Eco*RI and *Xho*I restriction sites, introduced by the primers, were used to clone *PHO87* into the corresponding sites of YEp352-PGK (yeast episomal plasmid carrying the promoter and terminator regions of *PGK1*). The resulting construct, YEp-*PHO87* (Figure 11) was transformed into yeast according to the lithium acetate method described by Ausubel *et al.* (1994). Transformants were selected on media lacking leucine.

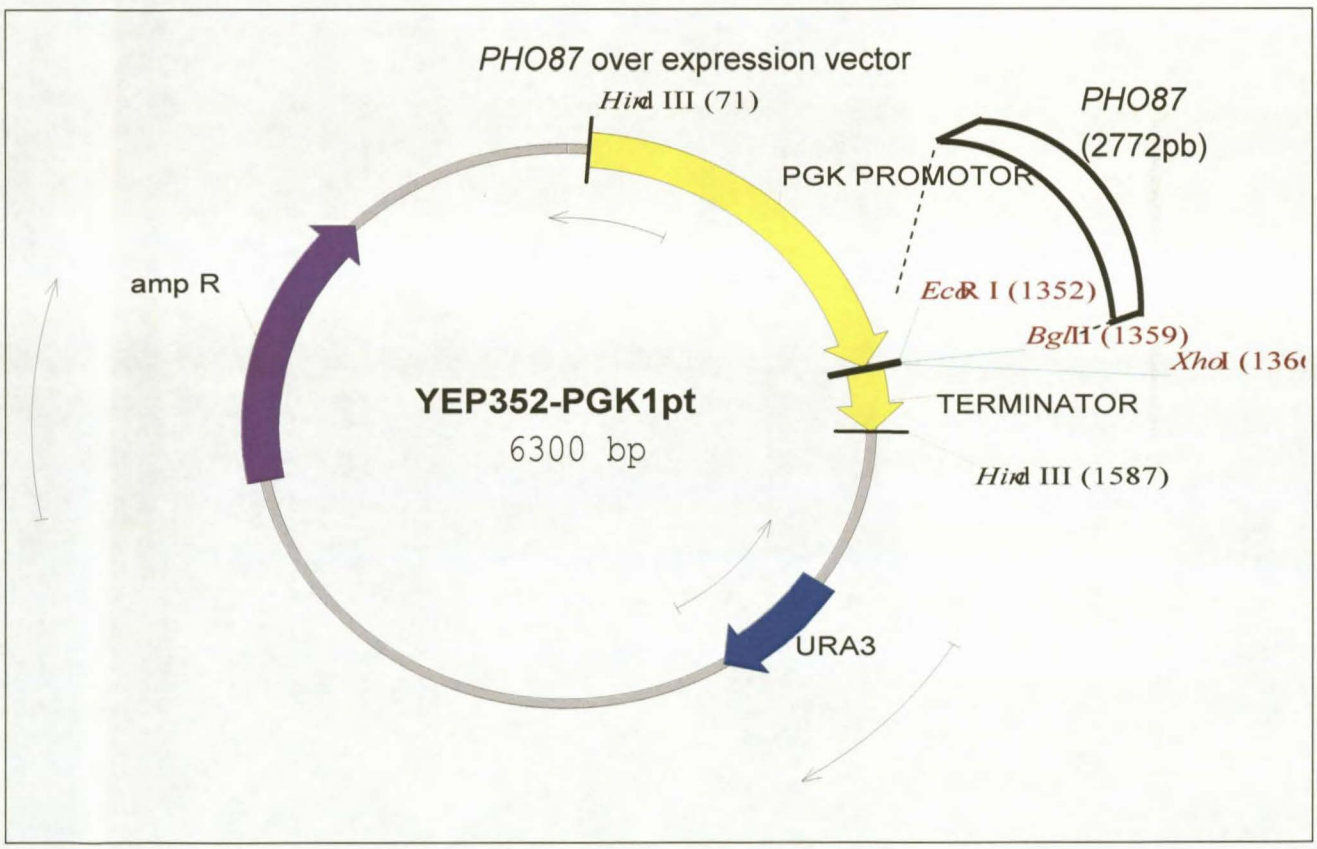


Figure 11. Schematic representation of YEp-*Pho87* plasmid.

3.2.4.3 INVASIVE GROWTH AND PSEUDOHYPHAL DIFFERENTIATION ASSESSMENT

Three colonies of *Yarrowia lipolytica*, *Pichia pastoris* and the different *S. cerevisiae* strains, PSY142, ISP15, W303 and L-5366, were grown individually at 30°C in standard YNB,

YPD, as well as YNB and YPD supplemented with 10 mg/L and 100 mg/L of carnitine. Cultures were grown to an $O.D._{600}$ of 1.0, and 10 µl of the cell suspensions was dropped onto SLAD medium containing 10mg/L and 100 mg/L of carnitine. The plates were spotted in triplicate and were subsequently incubated at 30°C. After three, five and ten days, the yeast colonies were removed from the plates under running water. Invasive cells remain present in the solid media following the washing procedure. The ability of the various strains to form cell filaments (pseudohyphae) and cellular morphology were assessed under the 10X magnification of a Nikon Optiphot-2light microscope.

3.2.4.4 HYPEROSMOTIC AND TEMPERATURE STRESS ASSAYS

A physiological role for carnitine was assessed under various stress conditions. In general, three colonies of each strain were grown in liquid YNB and YPD to serve as precultures. Subsequently 5 ml of liquid YNB and YPD, as well as corresponding media supplemented with concentrations of carnitine as indicated for each experiment were inoculated to an $O.D._{600}$ of 0.05. Cultures were grown to an $O.D._{600}$ of 1.0 and 15 µl of each cell suspension, as well as 10^{-1}, 10^{-2}, and 10^{-3} dilutions (in water) thereof, were spotted in triplicate onto YNB and YPD plates containing carnitine, sorbitol and sodium chloride concentrations as indicated.

To assess the correlation between carnitine levels and resistance to osmotic stress, the dilution series were spotted onto plates containing between 0.8M to 2.5M sorbitol and sodium chloride, as indicated in the figures. Plates were incubated at 30°C for 5 to 10 days, depending on the intrinsic growth rate and resistance of the yeast species that were analysed.

Similar to the osmotic stress analysis, heat shock resistance was assessed on YNB and YPD plates containing the same concentrations of carnitine as for the osmotolerance assays. Dilution series of strains were spotted in triplicate onto three sets of the above mentioned media. The three plate sets were incubated either at 30°C (the untreated control), or for 30 min at 55°C (heat shock), or for 15 min at 42°C followed by 30 min at 55°C (heat shock following a pre-adaptation period). All the plates were subsequently incubated at 30°C for five to ten days depending on the growth rate of the yeast strain.

3.2.4.5 LIQUID GROWTH ASSAYS

The growth rates of wild type FY23 and of an isogenic mutant lacking functional copies of the three known carnitine acetyl transferases encoding genes *YAT1*, *YAT2*, and *CAT2* were assessed in liquid medium containing carnitine or acetyl-carnitine. The precultures were grown in YNB to an $O.D._{600}$ of 1.0 for two consecutive rounds. Following the preculture, YNB media containing NaCl, carnitine or acetyl carnitine at the indicated

concentrations were inoculated to an initial $O.D._{600}$ of 0.05 and grown at 30°C. Growth curves were created for the individual strains by taking optical density readings at three hour intervals over a period of eight days.

3.2.4.6 IDENTIFICATION AND ANALYSIS OF CARNITINE RELATED GENES

Proteins involved in the *E. coli* carnitine pathway were blasted against the *Saccharomyces cerevisiae* protein database.

Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the corresponding *S. cerevisiae* mutants obtained from the BY4742 mutant library (Euroscarf) were exposed to the previously described osmotic shock procedure, and the plates were incubated aerobically and anaerobically for a week at 30°C.

3.2.4.7 HPLC-ELECTROSPRAY MASS SPECTROMETRY ANALYSIS

Yeast cultures were prepared according to the procedure described for the physiological liquid assay. Approximately 10^8 cells were collected, washed three times, suspended in 500µl of sterile water and broken by micro-glass beads. Samples were centrifuged, 100 µl of the supernatant suspended in 900 µl of acetonitrile and centrifuged again prior to HPLC-Electrospray-MS/MS-spectrometry analysis. Measurements were performed according to the method described in the appendix.

3.2.4.8 RADIOACTIVELY LABELED SAMPLE PREPARATION AND PAPER CHROMATOGRAPHY

Triplicate samples of FY23 and W303 were grown aerobically and anaerobically at 30°C to $O.D._{600}$ of 1.0 in YNB and YNB containing 1.8 M NaCl and supplemented with 1mM uniformly labelled radioactive ^{14}C-carnitine. Following growth, 10^8 cells of each culture were collected, washed three times, suspended in 100µl of sterile water and broken by micro-glass beads. After centrifugation, 50 µl of the supernatant was loaded onto Whatman paper N°1 (Whitman Ltd., England), dried and migrated with propan-1-ol/glacial acetic acid/water (v/v/v 8/1/1) according to the method described by Lewis and Bieber (1979) for 16 hours. Radioactive carnitine and acetylcarnitine served as markers. After migration the paper was dried and incubated at -80°C with and X-ray film, developed and analysed.

3.2.5 DISCUSSION

In eukaryotic organisms, carnitine has been associated with fatty acid metabolism and the transfer of activated acetyl residues across intracellular membranes. Other roles have been suggested due to the many physiological effects of dietary carnitine complementation. Here we show that L-carnitine, similar to its role in some prokaryotes,

can protect yeast cells against hyperosmotic stress. More surprisingly, we also observe a protective effect on heat-shocked cells. In the case of hyperosmotic stress, the data suggest that carnitine acts as a compatible solute. In these conditions, only L-carnitine, and not D-carnitine presents a protective effect, and the effect is dosage-dependent for low concentrations of carnitine, reaching a maximum protection at concentrations of between 100 and 200 mg/L.

It is more difficult to provide an explanation for the protective effect of L-carnitine in high temperature stress conditions. Indeed, the data show a clear L-carnitine-related phenotype, but require further confirmation and should be considered as preliminary.

To unravel the molecular mechanisms behind the protective effects of carnitine, several mutant strains were investigated. In a first round of assessments, genes that are associated with carnitine-dependent metabolic activities were investigated. The data clearly suggest that the genes involved in the carnitine shuttle and the transfer of activated acetyl residues between CoA-SH and carnitine are not required for osmo-protection. This furthermore indicates that the protective compound is carnitine and not an acyl-derivative thereof. In this regard, the data indicating that carnitine can not be metabolised to any other compound by yeast provide further support for the hypothesis that carnitine itself acts as a protective compound.

Deletion of the carnitine transporter-encoding *AGP2* gene diminished, but did not abolish, the protective effect of carnitine. Agp2p has been shown to be down-regulated under osmotic stress conditions in a Hog1 MAP kinase cascade depending mechanism (Lee *et al.*, 2002). The authors reported diminished levels of cellular carnitine in such conditions, but did not assess whether the presence of carnitine had a positive impact on the osmotic stress response. In addition to transporting carnitine, Agp2p is a carrier of polyamines (Aouida *et al.*, 2005) and amino acids (Schreve and Garrett, 2004), and the down-regulation in response to osmotic stress may be a response to those additional carrier activities. Our data do not contradict those finding, and suggest that another transporter is able to transport carnitine. The investigation shows that the *PHO87* gene may be such a carrier. The gene was investigated because preliminary studies on genes that would suppress the *cit2*Δ*cat2*Δ growth defect on non-fermentable carbon sources had led to the cloning of this gene, which imparted a partially suppressed phenotype when present in multiple copies (data not shown).

Our data indicate that *PHO87* expression from a multiple copy plasmid leads to improved stress resistance, and that this improvement is dependent on the presence of carnitine. On the other hand, a *PHO87* deletion strain does not show improvement in osmotic stress resistance in the presence of carnitine.

The data furthermore demonstrate that the improved stress resistance correlates with increased levels of carnitine in the cells. Indeed, the strain expressing the *PHO87* gene from a multiple copy plasmid contains approximately twice the amount of carnitine than the corresponding wild-type strain, while the deletion strain shows a two-fold reduction from this level.

PHO87 encodes a membrane protein that has been identified as a low-affinity inorganic phosphate carrier (Bun-ya *et al.*, 1996). The molecular role of the protein in carnitine-dependent stress resistance can not be clearly established from the data. However, two hypotheses can be proposed. Firstly, Pho87p may itself act as a carnitine carrier. However, inorganic phosphate is negatively charged, while carnitine is positively charged. Furthermore, the molecular structures of phosphate and carnitine are very different. While therefore not impossible, it seems unlikely that the same protein would act as a carrier for both molecules. In a second hypothesis, Pho87p could act as a facilitator, activating or inactivating other factors responsible for carnitine uptake. Both carnitine and phosphate are important for energy metabolism, and an indirect interaction between transporters to favour uptake of one or the other, while not having been demonstrated, may occur.

Interestingly, our Blast searches show that the protein most homologues to Pho87p in the human proteome is SLC13A2, which has been defined as a transporter of small molecules and is involved in osmo-regulation.

The data presented here require additional confirmation, and should be considered as preliminary. Nevertheless, they clearly establish that carnitine can act as a stress protectant in eukaryotic cells similar to its role in some microorganisms such as *Listeria monocytogenes* (Angelidis and Smith, 2003). It also clearly shows that this role is due to the molecule of carnitine itself, and not to derivatives thereof, and that genes involved in the carnitine shuttle are not required for this role. The contribution of individual genes to the newly described phenotype will have to be further investigated before final conclusions can be drawn. It is nevertheless clear hat *PHO87* does play a role in the process.

3.2.6 ACKNOWLEDGEMENTS

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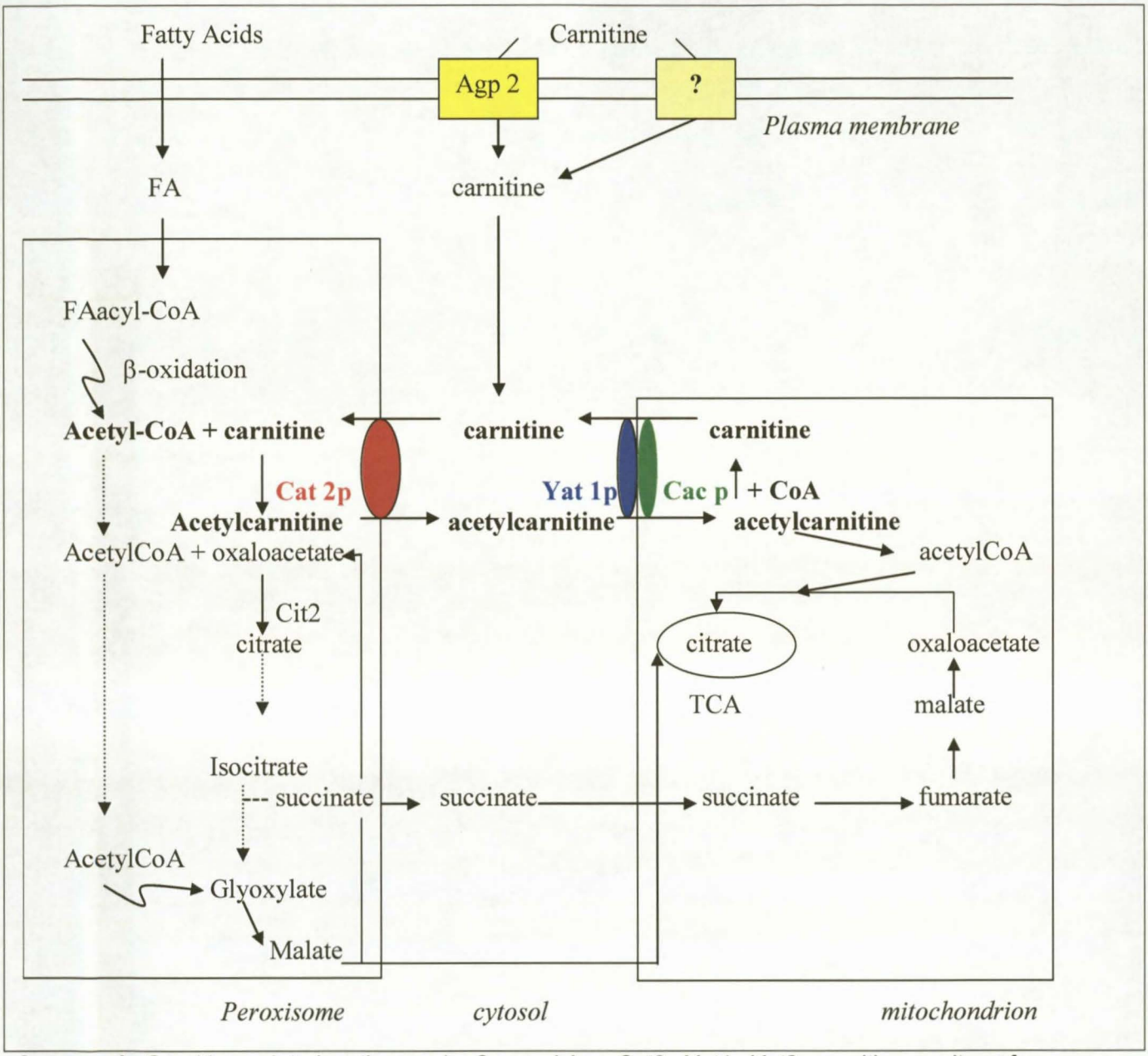
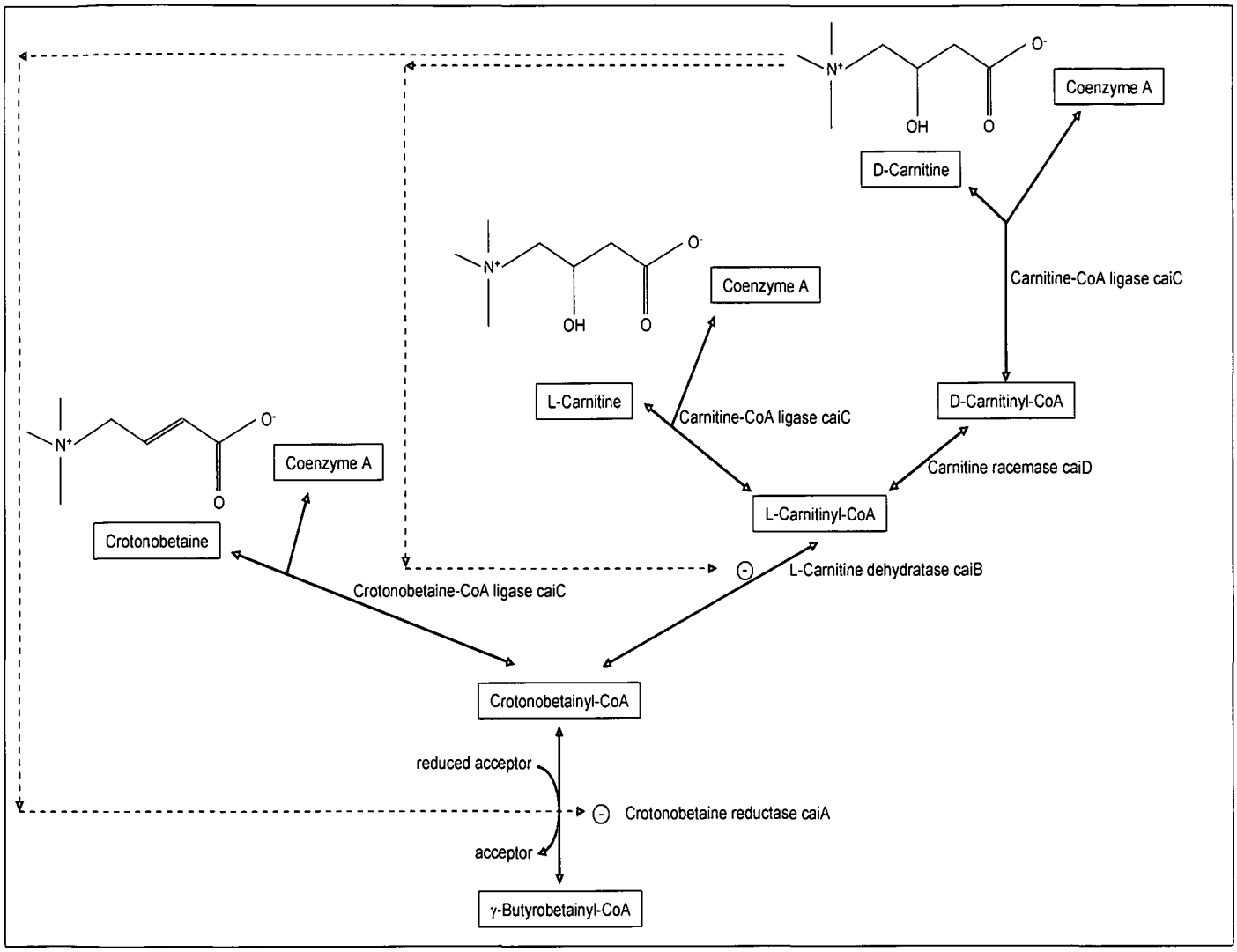
ANNEXE 1



Annexe 1. Carnitine related pathways in *S.cerevisiae*. Cat2, Yat1, Yat2: carnitine acyltransferase, Agp2p: general amino acids transporter allows carnitine uptake, Cac: translocase, Cit2: Citrate synthase.

ANNEXE 2



Annexe 2. Carnitine pathways in *E. coli*. CaiA (crotonobetaine reductase), CaiB (L-carnitine dehydratase), CaiC (crotonobetaine/carnitine CoA ligase), CaiD (carnitine racemase).

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

4.1 CONCLUDING REMARKS

In higher eukaryotes, and in particular *H. sapiens*, severe stress as experienced during serious illness, surgery, sepsis, burns or other critical states, is resulting in catabolic changes and hypermetabolism (Weissman, 1999). Metabolically these changes can be roughly characterized as glucose and lipid metabolism alteration (Wolfe, 1996; Kupari *et al.*, 1998). Increased lipolysis and glucose production are a response to the increased need for energy of stressed cells (Chiolero *et al.*, 1997). As carnitine is known to increase glucose oxidation, to balance CoA-levels and to be a part of fatty acid metabolism, it is no surprise that carnitine supplementation can be beneficial to these patients, and that the improved general state of patient may be due to the increased amount of available energy. But oxidative stress studies carried out on rats also showed that carnitine appears to protect chaperone activity to avoid protein aggregation and to reduce oxidative stress-induced post transcriptional modifications of proteins (Peluso *et al.*, 2001). Carnitine supplementation in hemodialyzed patients helps to ameliorate lipid metabolism, as expected, but also increases red blood cells numeration (Vesela *et al.*, 2001). L-carnitine was also shown to improve bone mineralization (Benvenga *et al.*, 2001), to have beneficial effects on HIV patients, reducing lymphocytes apoptosis (Famularo *et al.*, 1994), and improving CD4 cell counts and HIV loads (Cifone *et al.*, 1997). It has also been found to help in the prevention of damaging effects of AZT, as AZT causes structural and functional damages in the mitochondria. Carnitine appears to interact with mitochondrial membranes and induces changes in membrane permeability and helps the protection and recovery mechanisms of mitochondria (Monti *et al.*, 1994).

Such clinical and experimental research support the hypothesis that carnitine may have other properties and be a part of other cellular or molecular functions. The objective of this study therefore was to determinate if L-carnitine has other effects on the physiology of eukaryotic cells which may help explain the improvement seen in patients who are under acute stress.

In the opening chapters of this document, the role of carnitine in various organisms is discussed. The chapters highlight that, while many of the molecular functions of carnitine have been elucidated over the last few decades, many aspects of the role of carnitine and of its metabolism, as well of the role of specific genes involved in carnitine-dependent metabolic activities, remain not clear. To investigate whether carnitine can play additional roles, a well understood as well as easily manipulated model systems such as the yeast *S. cerevisiae* offers unique tools and opportunities. However, when taking such an

approach, there is an obvious risk that nothing may turn up, that the search for new roles may remain unsuccessful. In our case, the search was facilitated since information on prokaryotic systems was available. In prokaryotes, carnitine plays diverse roles as carbon or nitrogen source or as a stress protectant. The literature review of this thesis therefore presented an overview of the various roles of carnitine across the evolutionary range of existing organisms.

Guided by these existing data, the investigation focused on the role of carnitine in cellular adaptations to environmental factors. In a first set of data, we confirmed that carnitine was not used as a nutrient by yeast, and that it was not involved in inducing specific cellular adaptations to nutrient limitation, including invasive and pseudohyphal growth.

Our search was successful when investigating possible roles of carnitine in stress protection. Stress can be defined as a "physical, chemical, or emotional factor that causes bodily or mental tension and may be a factor in diseases causation", and as a "state of bodily or mental tension resulting from factors that tend to alter an existent equilibrium" (Medline dictionary). All living organisms are continuously exposed to physical and chemical stresses, which include oxidative stress (exposure to free oxygen radicals), osmotic shock (changes in external osmotic pressure), temperature stresses (change in temperature) and others. Our data clearly indicate that carnitine can act as a osmotic stress and temperature stress protectant in eukaryotic cells, similar to the role described in some prokaryotic organisms such as *Listeria monocytogenes* (Angelides and Smith, 2003). However, the data may appear to be contradicting evidence presented by Lee *et al.* (2003) who showed that osmotic stress leads to a decrease in *AGP2* expression and carnitine uptake. These authors indicated that *AGP2* down-regulation was mediated via the Hog1 MAP kinase pathway, and showed that *agp2* mutants were more salt-tolerant than wild type strains. However, the authors did not investigate the specific effect of carnitine on stress resistance. Since Agp2p is responsible for the transport of a variety of compounds, including many amino acids and polyamines (Schreve and Garrett 2004; Aouida *et al.* 2005), the down-regulation of this gene may be linked to its involvement in such additional systems. Indeed, it is not clear why cells would restrict the uptake of compounds that can balance hyperosmotic conditions, as is the case of carnitine.

This work is the first report of carnitine acting as a stress protectant in yeast. As such, the investigation opens new avenues of research, and may again provide proof for the usefulness of *S. cerevisiae* as a model system. Nevertheless, it is also clear that many of the data require further confirmation, and must be considered as preliminary.

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APPENDIX

THE DETERMINATION OF CARNITINE ACETYLTRANSFERASE ACTIVITY IN *SACCHAROMYCES CEREVISIAE* BY HPLC-ELECTROSPRAY MASS SPECTROMETRY

APPENDIX

The Determination of Carnitine Acetyltransferase Activity in *Saccharomyces cerevisiae* by HPLC-Electrospray Mass Spectrometry

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SUMMARY

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

INTRODUCTION

Carnitine acetyltransferases (EC 2.3.1.7) are present in eukaryotic organisms and have been described in yeast, fungi, plants and mammals (Burgess and Thomas, 1986; Kozulic *et al.*, 1987; Stemple *et al.*, 1998; Bieber, 1988; Jernejc and Legisa, 1996; Masterson and Wood, 2000). They catalyse the transfer of activated acetyl groups from acetyl-CoA to L-carnitine or from acetylcarnitine to CoA-SH. This reaction is required because organellar membranes are impermeable to acetyl-CoA (Van Roermund *et al.*, 1995), while a carnitine-acetylcarnitine antiport system can ensure the transfer of the activated acetyl group without loss of energy (Palmieri *et al.*, 1999; Van Roermund *et al.*, 1999).

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

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

quadropole mass spectrometer was set to pass the precursor ion (acetylcarnitine) into the fragmentation cell, where collision-induced dissociation created the *m/z* = 85 fragment, which was detected by the second analyser. The combination of both HPLC and MS/MS provides a highly selective and specific method for the detection and quantification of acetylcarnitine in the presence of high concentrations of L-carnitine. No extraction steps are required in this assay, contributing to the simplicity and accuracy of the method.

MATERIAL AND METHODS

YEAST STRAINS AND PLASMIDS

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

Table 1 Strains and plasmids used in this study

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

CLONING AND DISRUPTIONS OF GENES

To create a yeast strain without any known CAT-encoding genes, the *CAT2* and *YAT1* genes were deleted from strain FY23Δ*yat2* (Swiegers *et al.*, 2001). A 595 bp *Bam*HI/*Bgl*II fragment internal to the *CAT2* gene of the plasmid PGEM-T-easy-*CAT2* was replaced by a 1.1 kb *URA3* gene from plasmid YDp-U. The resulting construct, pΔ*cat2*::*URA3*, was used to isolate a 2.9 kb disruption cassette containing the *URA3* gene plus *CAT2* flanking regions, which was then transformed into FY23Δ*yat2*. Transformants were isolated on selective media and the disruption was verified using the primers CAT2F1 and CATR1. The Δ*yat1* disruption construct was created using the plasmid pΔ*yat1*::*LEU2* and replacing the *LEU2* gene by removing it as a 1.6 kb *Bam*HI fragment and replacing it with a 0.8 kb *TRP1* fragment from plasmid YDp-W. The resulting disruption construct, pΔ*yat1*::*TRP1*, was used to isolate a 1.9 kb fragment containing the *TRP1* gene and *YAT1* flanking regions, which was transformed into strain FY23Δ*yat2*::*LEU2* Δ*cat2*::*URA3*. The disruptions were verified using primers YAT1F1 and YAT1R1.

CULTIVATION AND HOMOGENISATION OF YEAST

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

CARNITINE ACETYLTRANSFERASE ASSAY

The assay mixture consisted of a buffer containing 13.5 mM KCl and 4.5 mM TRIS (pH 7.4) complemented with 0.049 mM acetyl-CoA and 2.47 mM L–carnitine. After a preincubation of 10 min at 30°C, the reaction was initiated by the addition of 10 µl of cell extract with a concentration of 1.3 mg/l total proteins. To measure the formation of acetylcarnitine, samples were taken at 10 min intervals by removing 100 µl aliquots from the sample mixture. These were added to 900 µl acetonitrile, thereby stopping the reaction. The samples were kept on ice and then deproteinised by centrifugation at 12 000

rpm for 5 minutes. The supernatants were transferred to new tubes and analysed by HPLC/MS/MS.

MASS SPECTROMETRY

Mass spectrometry was performed on a Micromass (Manchester, UK) Quattro triple quadropole mass spectrometer fitted with an electrospray ionisation source. Solvent A (acetonitrile/water/formic acid: 30/70/0.05(v/v/v)) was used as carrier solvent and was supplied to the ionisation source by an LKB/Pharmacia (Sweden) pump. For direct injection of the acetyl carnitine standard, the flow rate was 20 µl/minute and 5 µl of the standard was injected through a Rheodyne injection valve. The molecular ion ($[M+H]^+$) of acetylcarnitine was observed using a capillary voltage of 3.5 kV, a source temperature of 80°C and a cone voltage setting of 20 V. To obtain the fragment pattern of acetylcarnitine, the molecular ion was dissociated in the fragmentation cell by collision-induced dissociation at an argon pressure of 2.8 $X10^{-3}$ mbar, applying a collision energy of 20 eV. The resultant fragments were scanned in the second analyser.

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

RESULTS

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

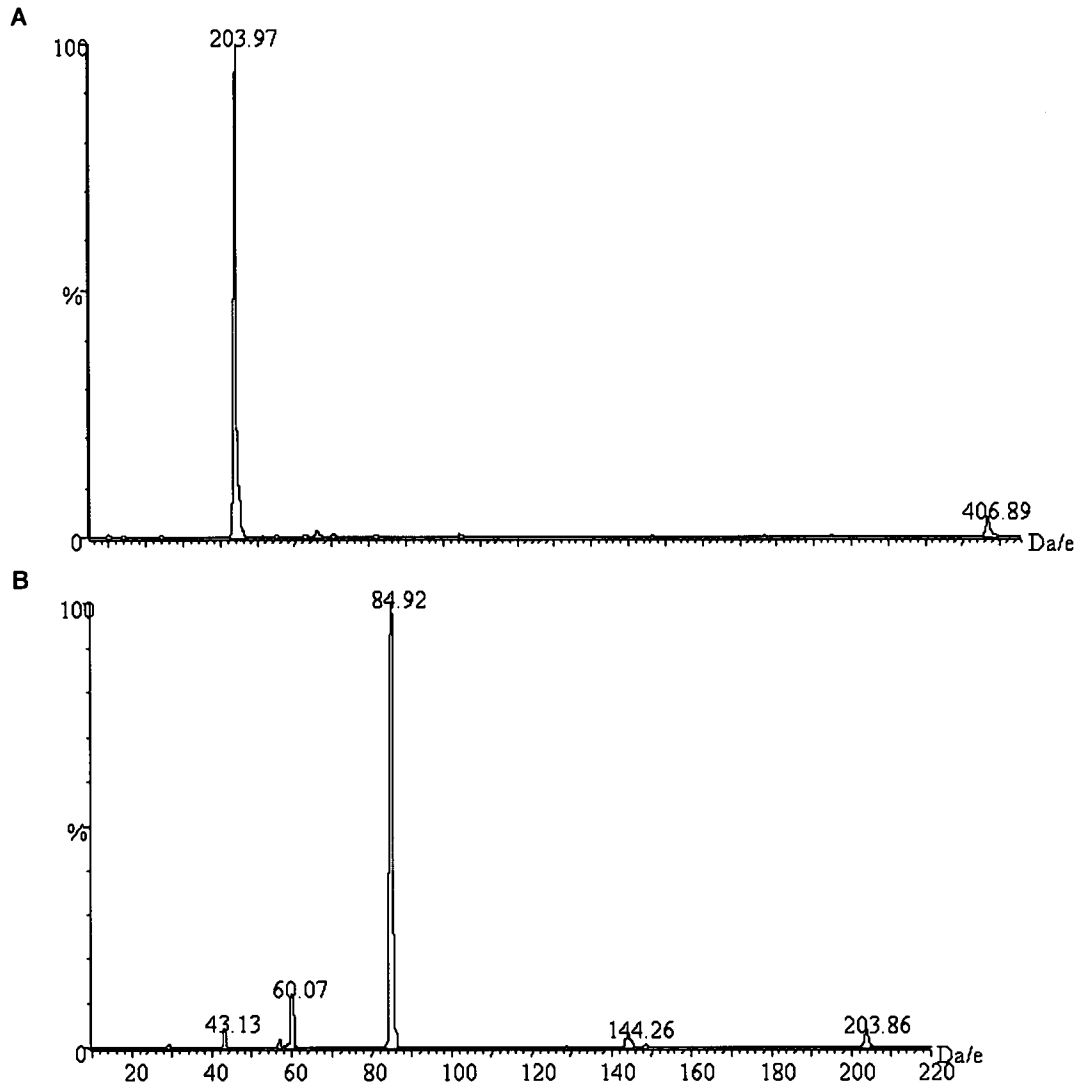

Figure 1. Electrospray mass spectrum of the parent ion from acetylcarnitine (**A**) at m/z = 204. The fragmentation resulted in the fragmentation ion at m/z = 84 (**B**), which was used for detection.

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

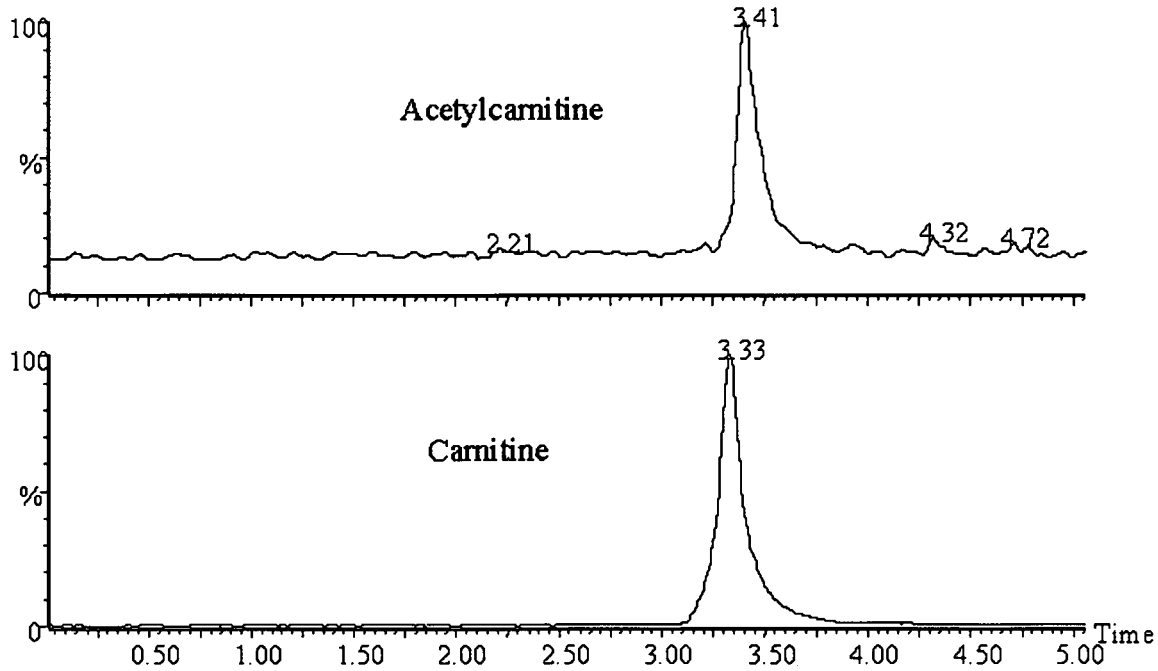

Figure 2. HPLC chromatogram of the separated components in the reaction mixture. The figure clearly indicates that there is no crossing over.

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

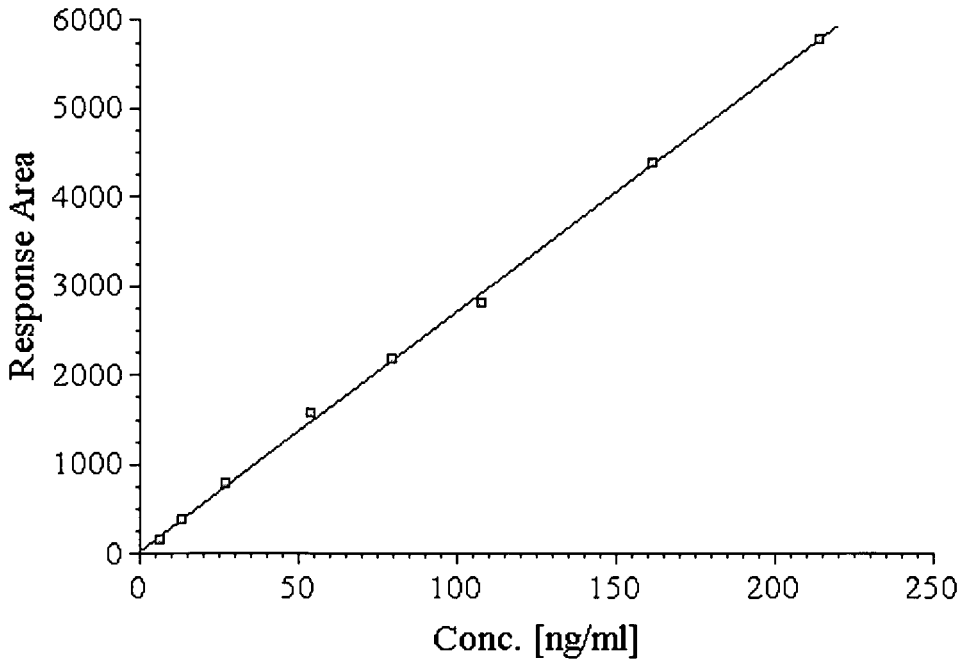

Figure 3. The calibration graph of acetylcarnitine-hydrochloride. The standard curve was used to determinate the concentration of acetylcarnitine formed during the enzyme assay.

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

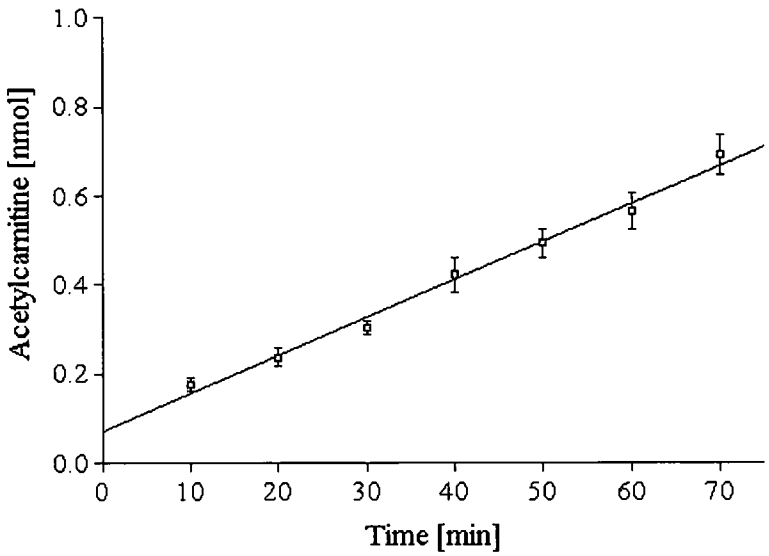

Figure 4. Time-dependent formation of acetylcarnitine due to the presence of CAT enzymes in the yeast crude extract. The reaction was performed as described under materials and methods. The graph presents the average of six repetitions.

In a second set of experiments, the effect of various protein concentrations on the formation of acetylcarnitine was measured. Here too, a near linear increase in product formation for increasing protein concentration over a fixed period of time was observed. The reaction was stopped either after 20 min (Fig. 5A) or after 40 min (Fig. 5B).

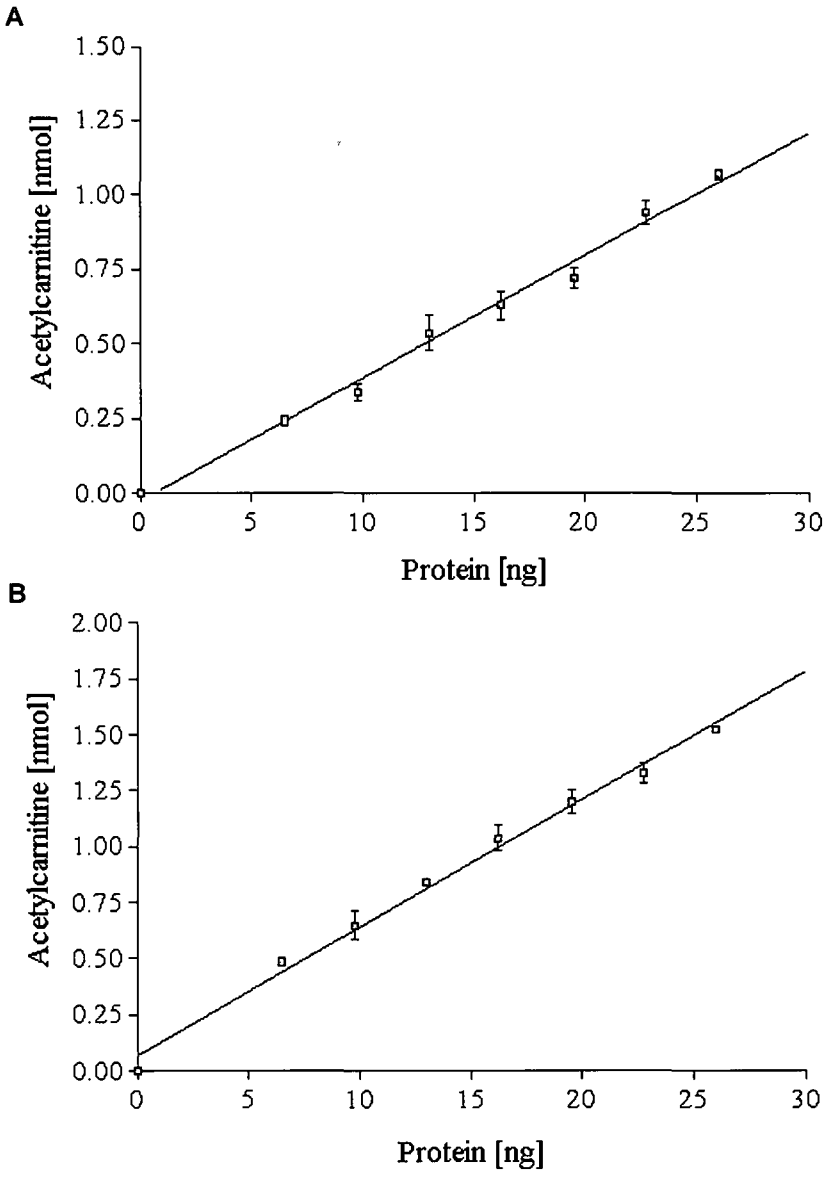

Figure 5. Protein-dependent formation of acetylcarnitine due to the presence of CAT enzymes in the yeast crude extract. The reaction was performed with different protein concentrations and was stopped either after 20 min (**A**) or after 40 min (**B**). The graph presents the average of six repetitions.

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

DISCUSSION

The yeast *S. cerevisiae* is an important model organism used to study molecular processes in eukaryotic cells. Due to the ease of genetic manipulation, this organism has contributed greatly to advancing the understanding of the molecular mechanisms involved

in metabolic processes. In order to have a better understanding of the role of each of the CAT enzymes in the metabolism of yeast, it is important to study the activity of these enzymes collectively and individually in a wide range of physiological conditions. An assay was developed, based on the time-dependent formation of acetylcarnitine from free carnitine and acetyl-CoA. Acetyl carnitine was measured by HPLC in combination with tandem mass spectrometry.

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

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

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