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REDESIGN OF CARNITINE ACETYLTRANSFERASE SPECIFICITY BY PROTEIN ENGINEERING

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MATERIALS AND METHODS

1. MATERIALS

1.1. ANIMALS

The following animal species were used in the present study:

A) Female New Zealand White (NZW) rabbits (*Oryctolagus cuniculus*).

B) Sprague-Dawley rats (*Rattus Norvegicus*)

All the experimental protocols with animals were approved by the Ethics Committee for animal experimentation of the University of Barcelona.

1.2. BACTERIAL STRAINS

Two bacterial strains of *Escherichia coli* were used in this study: DH5α and BL21.

Strain	Genotype	Reference
DH5α	*sup* E44, Δ*lac*U169 (ϕ80 *lac* ZΔM15), *hsd* R17, *rec* A1, *gyr* A96, *thi*-1, *rel* A1	Stratagene
BL21	F-, *omp* T, *hsd* S_B (r_{B-}, m_{B-}), *dcm*, *gal*, λ(DE3), pLysS, Cm^r	

1.3. YEAST STRAINS

The following *Saccharomyces cerevisiae* strains were used in this study:

Strain	Genotype/strain description	Reference
FY23	*MATa trp1 ura3 leu2*	Winston, 1995
FY23Δ*cat2*	*MATa trp1 ura3 cat2::LEU2*	Swiegers, 2001
YPH499	*MATa ura3-52 leu2-D1 ade2-101 lys2-801 his3-D200 trp1-D63*	
VIN 13	Commercial wine yeast strain	Anchor yeast

A *S. cerevisiae* strain (FY23Δ*cat2*) devoid of COT and CPT activity and lacking the endogenous *CAT2* gene was used as an expression system (Swiegers, 2001). Although this strain conserves two additional CrAT genes (*YAT1* and *YAT2*), its carnitine acetyltransferase activity in the conditions in which it was expressed in this study was not detected.

1.4. PLASMID VECTORS

The following plasmid vectors were used for cloning strategies:

A) pGEM®-T Vector

The pGEM®-T Vector System (Promega, ref. A3600) is a convenient system for the cloning of PCR products. This vector (3 kbp) presents 3´ terminal thymidine overhangs at the insertion site which greatly improve the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases.

B) pYES 2.0 plasmid vector

The pYES 2.0 (Invitrogen, ref. V825-20) is a high-copy episomal vector of 5.9 kbp designed for high-level expression of recombinant proteins in *S. cerevisiae* under the galactose promoter *GAL1*. It contains the *URA3* gene for selection in yeast and 2 micron origin for high-copy maintenance.

C) pGEX-6P-1 vector (Glutathione S-transferase (GST) gene fusion system)

The GST gene fusion system is an integrated system for the expression and purification of fusion proteins in *E. coli*. The pGEX-6P-1 vector (Fig. 1) (Amersham Biosciences, ref. 27-4597-01) is designed for inducible, high-level intracellular expression of genes in *E. coli* as fusions with *Schistosoma japonicum* GST (26 kDa).

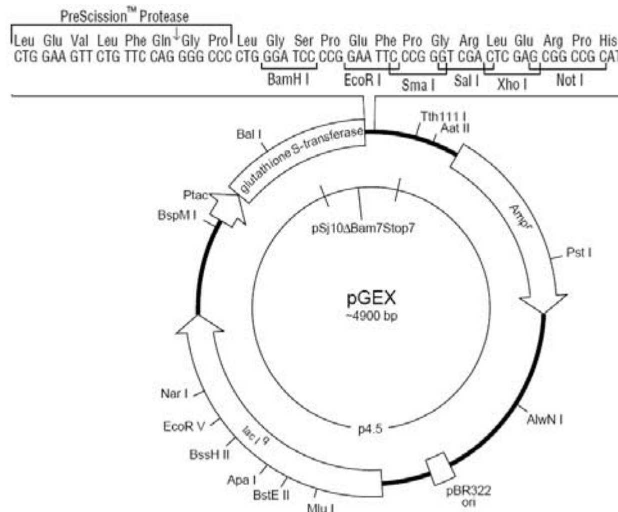


Fig. 1**. Map of the pGEX-6P-1 vector (4.9 kbp) showing the reading frames and main features.**

D) <u>pHVX2 plasmid</u>

The pHVX2 plasmid (Fig. 2) is a multicopy episomal plasmid of 7.5 kbp designed for high-level expression of recombinant proteins in *S. cerevisiae* under the control of the constitutive *S. cerevisiae* phosphoglycerate kinase gene (*PGK1)* promoter and terminator (Volschenk, 1997). The plasmid contains the restriction sites *Eco*RI, *Xho*I, and *Bgl*II located in between the *PGK1* promoter and terminator. It also contains the *S. cerevisiae LEU2* gene for complementation of auxotrophic strains. The plasmid also contains the 2 micron origin which results in multiples copies per cell of the vector.

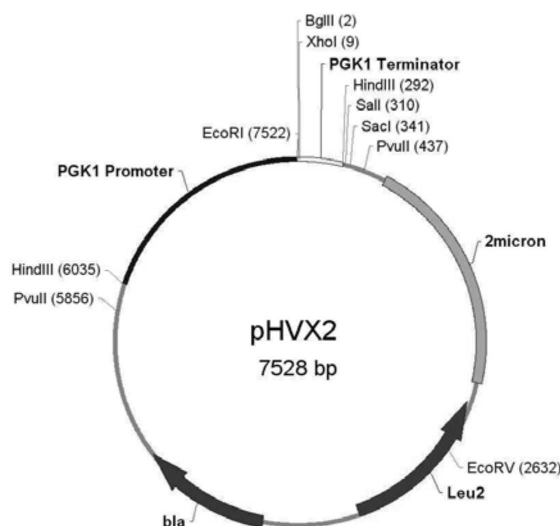


Fig. 2. **Map of the pHVX2 vector showing the main features.**

1.5. OLIGONUCLEOTIDES

The oligonucleotides used in this study were all purchased from Sigma-Genosys. Oligonucleotide names and sequences are listed in the Appendix.

2. DNA AND RNA BASIC TECHNIQUES

2.1 BACTERIAL CULTURE

Bacterial strains are cultured in LB medium (Luria-Bertrani Broth) in the presence of the appropriate antibiotic. In the case of the bacterial strains and vectors used in this study, the antibiotic ampicillin was used. Ampicillin binds to and inhibits a number of enzymes in the bacterial membrane that are involved in the synthesis of the cell wall.

The ampicillin-resistance (*amp*^{r}) gene carried on the plasmid or on the bacterial strain genome codes for an enzyme that is secreted into the periplasmic space of the bacterium, where it catalyzes hydrolysis of the β-lactam ring, with concomitant detoxification of the drug. Therefore, only the cells with the *amp*^{r} gene present on their genome or in the target plasmid will survive in a medium containing this antibiotic.

LB medium: 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl dissolved in distilled water. It is autoclaved immediately and kept at room temperature (r.t.). 100 mg/L ampicillin prepared in water is added at the moment of use.

Solid LB medium: LB medium plus 2% (w/v) agar. It is autoclaved immediately. When the temperature is about 50 ºC, 100 mg/L ampicillin is added and the medium is distributed into 10-cm plates with 30 ml of medium each. Plates are stored at 4 ºC.

2.2. PLASMID DNA PREPARATION

Plasmid DNA could be obtained from bacterial cultures that contain the plasmid of interest by growing them in LB medium with the appropriate antibiotic and the later purification of the plasmid DNA from the cell lysate.

Minipreparations of plasmid DNA were obtained using the *Minipreps DNA Purification System* (Promega, A1460) where the yield of high-copy-number plasmids obtained is about 3-5 µg DNA per millilitre of the original bacterial culture. When higher amounts of plasmid DNA were needed the *QIAGEN Plasmid Maxi kit* (QIAGEN, ref. 12163) was used. In this case up to 500 µg DNA could be obtained.

Minipreparations are obtained from 2 ml LB medium with the appropriate antibiotic which has been inoculated with a single colony of *E. coli* cells and grown overnight (o/n) at 37 ºC with moderate shaking. For maxipreparations, 500 ml of LB medium with the appropriate antibiotic is inoculated with 1 ml of the starter culture and grown o/n at 37 ºC with moderate shaking. In both cases cells are harvested by centrifugation and lysed with NaOH and SDS. The precipitate formed contains the bacterial genomic DNA, proteins, cell debris and SDS. The bacterial RNA is degraded by the action of RNase A. Then, the plasmid DNA is purified from the supernatant using ion-exchange resin columns. After that, the DNA is washed and eluted, and it is pure enough for enzymatic modifications, PCR, sequencing, etc.

For future preparation of plasmid DNA, a stab of each construct is prepared as follows: 1 ml aliquot from the 500 ml maxipreparation inoculum is mixed with 500 µl 50% sterile glycerol and stored at -80 ºC.

2.3. DNA ENZYMATIC MODIFICATIONS

2.3.1. DNA digestion with restriction enzymes

Restriction endonucleases are enzymes isolated and purified from bacteria or fungi which bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition sequence. The most used restriction enzymes recognize specific sequences that are four, five or six nucleotides in length and display two-fold symmetry. The location of cleavage sites within the axis of dyad symmetry differs from enzyme to enzyme: some cleave both strands exactly at the axis of symmetry, generating fragments of DNA that carry blunt ends; others cleave each strand at similar locations on opposite sides of the axis of symmetry, creating fragments of DNA that carry protruding single-stranded termini (cohesive ends).

Each restriction enzyme requires specific reaction conditions of pH, ionic strength and temperature; therefore in each case the manufacturer's instructions were followed. In general, DNA is digested at a concentration of 0.5 µg/µl using 1-4 units (U) of the enzyme per µg of DNA. Restriction enzymes are stably stored at -20 ºC in a buffer containing 50% glycerol. Reaction volumes must be kept to a minimum by reducing the amount of water in the reaction as much as possible. However, the restriction enzyme must contribute less than 0.1 volume of the final reaction mixture; otherwise, the enzyme activity may be inhibited by glycerol. Digestions are carried out for 1 h in the specific buffer and the digestion products are analyzed in agarose gels.

When DNA is to be cleaved with two or more restriction enzymes, the digestions can be carried out simultaneously if both enzymes work well at the same temperature and in the same buffer. If the enzymes have different requirements, after the first digestion the DNA could be recovered using the *QIAquick PCR purification kit* (QIAGEN, ref. 28106). The DNA is selectively adsorbed to a silica-gel membrane and separated from enzymes, salts, etc. Then, the DNA is eluted in the desired volume of water and the second digestion or the next enzymatic modification is performed.

Enzyme digestion	
DNA	0.5 µg/µl
10X Buffer	1X
Enzyme	1-4 U/µg DNA

2.3.2. Blunt ends

The filling of 5'-protruding ends or the shortening of 3'-protruding ends allows the cloning by blunt-end ligation of non compatible restriction sites. The enzyme used for blunt end generation was the Klenow fragment of *E. coli* DNA Polymerase I (Roche, ref. 10 104 531 001). Klenow enzyme is a DNA-dependent DNA polymerase that carries 5' → 3' polymerase and 3' → 5' exonuclease activity, but lacks the 5'→ 3' exonuclease activity of the native enzyme. In the presence of deoxynucleosides triphosphate (dNTPs) this enzyme fills the gaps left by restriction enzymes that produce 5' protruding ends. In the absence of dNTPs the enzyme eliminates the 3' protruding end nucleosides. The reaction was done following the manufacturer's instructions.

2.4. DNA RESOLUTION AND PURIFICATION

2.4.1. DNA resolution in agarose gels

Electrophoresis through agarose gels is the standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid to perform and capable of resolving fragments of DNA from 50 bp to approximately 25 kbp in length on agarose gels of various concentrations. Higher fragments of DNA will need a lower concentration of agarose gel to be separated. For instance, 1% agarose gels are used to resolve DNA fragments between 0.5-7 kbp.

The location of DNA within the gel can be determined directly by staining the gel with low concentrations of the fluorescent dye ethidium bromide which intercalates between the two strands of the DNA. The presence of the DNA is visualized with ultraviolet (UV) light where the DNA fragment appears as a fluorescent orange band. Extra precautions should be taken because ethidium bromide is a powerful mutagen and is moderately toxic and UV radiation is dangerous, particularly to the eyes.

DNA which is negatively charged migrates to the anode of the electrophoresis chamber. This migration of the DNA is inversely proportional to its molecular weight logarithm and therefore, the molecular weight (MW) of a desired DNA fragment could be calculated by its interpolation on the regression curve of a molecular marker with known MW DNA fragments or by directly comparison with the molecular marker DNA fragments.

The agarose gel is prepared with low-melting-temperature agarose dissolved in 1X TAE electrophoresis buffer containing 0.5 μg/ml ethidium bromide (AppliChem Biochemica, ref. A1151,0001). Samples are loaded in 1X loading buffer and electrophoresis is run with 1X TAE electrophoresis buffer at 75 volts (V).

1X TAE electrophoresis buffer	
Tris-acetate pH 8.3	40 mM
EDTA	1 mM

5 X sample loading buffer: 30% sucrose, 0.2% orange G, 10 mM Tris-HCl pH 8.0, 40 mM EDTA.

5X sample loading buffer	
sucrose	15 g
orange G	100 mg
Tris-HCl 1M pH 8.0	0.5 ml
EDTA 0.5 M	4 ml
bd. H_2O up to	50 ml

2.4.2. DNA purification

To extract and purify a DNA fragment from the agarose gel the *QIAEX II Gel Extraction kit* (QIAGEN, ref. 20021) was used. Briefly, the agarose gel fragment containing the desired DNA is dissolved in a chaotropic agent and the DNA is selectively adsorbed into a silica resin which has been optimized to enhance recovery of 40 bp to 50 kbp DNA fragments. Impurities such as agarose, proteins, salts and other components of the sample are removed during washing steps. The pure DNA is eluted in water and is suitable for other manipulations.

2.5. DNA AND RNA QUANTIFICATION

RNA and DNA could be quantified in a spectrophotometer measuring the absorbance at 260 and 280 nanometers (nm) in 1 ml quartz cuvettes. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An optical density (OD) of 1 corresponds to approximately 40 µg/ml for single-stranded DNA and RNA, and 50 µg/ml for double-stranded DNA. The ratio between the readings at 260 and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of RNA and DNA have OD_{260}/OD_{280} values higher than 1.65 and 1.8, respectively.

2.6. DNA LIGATION

Ligation of a segment of foreign DNA to a linearized plasmid vector involves the formation of new bonds between phosphate residues located at the 5' termini of double-stranded DNA and adjacent 3' hydroxyl moieties.

The bacteriophage *T4 DNA ligase* was used for the ligation reactions following the manufacturer's instructions. The reaction is done in the presence of the ligation buffer and ATP for 2-3 h at 16 ºC in case of cohesive ends fragments and at 18 ºC o/n in case of blunt ends fragments. The ligation reaction should contain between 20-60 µg approx. of vector DNA per ml and an equal or slightly greater concentration of foreign DNA (insert) than of vector DNA.

When the ligation is done with different protruding termini DNA, the foreign DNA is inserted in only one orientation within the recombinant plasmid and the background of non recombinant clones is low. Ligation reactions involving blunt-ended molecules are much less efficient and require higher concentrations both of DNA ligase and plasmid and insert DNAs. Moreover, recombinant plasmids may carry tandem copies of foreign DNA and the background of non recombinant clones can be high.

Ligation was checked by PCR or by electrophoresis in an agarose gel before its transformation in competent *E. coli.*

2.7. PREPARATION AND TRANSFORMATION OF COMPETENT *E. coli*

2.7.1. Obtaining of competent *E. coli*

Competent cells are those cells which have been treated to increase their capacity to introduce a circular exogenous DNA. Competent cells could be purchased from a commercial source or prepared in the laboratory. In both cases, a yield of approx. 10^8-10^{10} transformed colonies/µg of supercoiled plasmid DNA could be achieved.

To obtain competent cells a single colony of *E. coli* cells is inoculated into 5 ml LB medium with the appropriate antibiotic and grown o/n at 37 ºC with moderate shaking. The next day, 500 ml of LB plus antibiotic is inoculated with 5 ml of the

preinoculum and grown for 3 h in the shaker at 37 ºC to an OD_{600} of 0.5-0.6. Cells are then chilled on ice and centrifuged at 4,000 x *g* for 20 min at 4 ºC. Cells should be kept at 4 ºC for the subsequent steps. After that, the pellet is immediately resuspended in 500 ml of sterile and ice-cold water and centrifuged again. This process is repeated and then the pellet is resuspended by swirling in remaining liquid. 40 ml of sterile, ice-cold 10% glycerol is added and the suspension is centrifuged at 4,000 x *g* for 10 min. Cells are resuspended in 500 µl of 10% glycerol and 40 µl aliquots are stored at -80 ºC.

2.7.2. Transformation of competent *E.coli*

The transformation process consists of introducing the plasmid DNA into the bacterial cells for later amplification and obtaining. Moreover, only the circular DNA will enter the cells, therefore if the DNA which is to be introduced into the cells is the result of a ligation, only the ligated plasmid will be obtained after the transformation.

Plasmid DNA was introduced by high-voltage electroporation (electrotransformation). In this technique, a high-voltage electric field is applied briefly to cells, apparently producing transient holes in the cell membrane through which plasmid DNA enters. Successful electroporation of *E. coli* requires long, strong pulses. Under these conditions up to 10^9 transformants/µg plasmid DNA can be achieved. The procedure was applied following the manufacturer's instructions (Eppendorf Electroporator 2510).

Electroporation settings: 2 mm gap cuvettes, 2.5 kV, which gives a 12.25 kV/cm field strength for 5-6 msec.

Electroporation procedure: 1 µl (~ 1 ng) of the DNA ligation reaction or control DNA is added to 40 µl of recently thawed competent cells, mixed and kept for 1 min on ice. After that, the cell mixture is placed on a chilled cuvette. The suspension must touch both side walls of cuvette. Immediately after the electroporation, a solution containing 1 ml of LB and 5 mM glucose is added to the cuvette, mixed thoroughly and incubated at 37 ºC for 1 h with moderate shaking. After that, 100 µl of the suspension is plated in LB plates with the appropriate antibiotic. Plates are incubated at 37 ºC o/n.

2.7.3. Recombinant plasmid selection

The bacteria which have incorporated the desired DNA are selected by plating the cells in LB plates with the appropriate antibiotic. After o/n incubation bacterial colonies are analyzed by PCR (PCR-Preps, Section 2.11.3) or by sequencing after growing them as described before for the DNA minipreparations (Section 2.2).

2.8. DNA SUBCLONING

Subcloning techniques are used for various purposes, such as the obtaining of protein expression constructs, isolation of mutated DNA fragments, etc. The protocol differs in each case although the basic DNA techniques described above were used. Original DNAs were modified enzymatically. The desired DNA fragments were purified and ligated and the resultant DNA construct was transformed, amplified and checked by PCR, sequencing or enzymatic digestion.

2.9. ISOLATION OF YEAST CHROMOSOMAL DNA

The following protocol was used for the isolation of yeast chromosomal DNA. A yield of 20 µg of chromosomal DNA should be obtained. The resulting DNA is ready to use for restriction digestion, PCR amplification or Southern Blot analysis.

1. Grow a 10 ml culture of yeast in YPD o/n at 30 ºC (see section 4.1)
2. Spin culture 5 min at 1200 x g, r.t.
3. Wash cells in bd. water.
4. Resuspend cells in 200 µl breaking buffer. Then, add the same volume of glass beads and 200 µl phenol/chloroform/isoamyl alcohol.
5. Vortex at highest speed for 3 min.
6. Add 200 µl TE buffer and vortex.
7. Centrifuge 5 min at high speed, r.t.
8. Transfer the aqueous layer to a new tube, add 1 ml of 100% ethanol and mix by inversion.
9. Centrifuge 3 min at high speed, r.t.
10. Resuspend pellet in 0.4 ml TE buffer.
11. Add 30 µl of 1 mg/ml DNase-free RNase A, and incubate 5 min at 37 ºC.

12. Add 10 µl of 4 M ammonium acetate and 1 ml 100% ethanol. Mix by inversion.
13. Centrifuge 3 min at high speed, r.t.
14. Discard supernatant and dry the pellet. Resuspend DNA in 100 µl sterile milliQ water.

Breaking buffer: 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
TE buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA.

2.10. RNA ISOLATION FROM TISSUES

RNA was obtained from rat tissues (testicles and heart) using the TRIzol Reagent (Gibco, ref. 15596-018). This reagent is a mono-phasic solution of phenol and guanidine isothiocyanates, which allows the isolation of total RNA, DNA and protein from cells and tissues.

RNA from tissues was extracted according to the manufacturer's instructions. Briefly, frozen tissues were powdered on liquid nitrogen with a mortar and a pestle, and then homogenized in 1 ml of TRIzol reagent per 50-100 mg of tissue. During sample homogenization or lysis, TRIzol Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase, DNA in the interphase and proteins in the organic phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol and dissolved in RNase-free water. Total RNA isolated by TRIzol Reagent is free of protein and DNA contamination and it is quantified using a spectrophotometer method (Section 2.5). It can be used for Northern blot analysis or molecular cloning, etc.

RNase-free water for RNA: RNase-free water is prepared in RNase-free glass bottles. Diethylpyrocarbonate (DEPC) 0.1% (v/v) is added to the water and solution is left to stand o/n and then autoclaved.

2.11. PCR DNA AMPLIFICATION

The polymerase chain reaction (PCR) is used to amplify a segment of DNA that lies between two regions of known sequence. Two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by a DNA polymerase. These oligonucleotides typically have different sequences and are complementary to sequences that lie on opposite strands of the template DNA and flank the segment of DNA that is to be amplified. The template DNA is first denatured by heating in the presence of a large molar excess of the two oligonucleotides and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. The cycle of denaturation, annealing and DNA synthesis is then repeated many times (between 25-35 cycles). Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product. The major product of this exponential reaction is a segment of double-stranded DNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose length is defined by the distance between the primers.

In 1988, a thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* (*Taq* DNA polymerase) was introduced (Saiki, 1988). Its optimal temperature for synthesis is 72 ºC. This enzyme, which can survive extended incubation at 95 ºC, is not inactivated by the heat denaturation step and does not need to be replaced at every round of the amplification cycle. *Taq* DNA polymerase is frequently used for routine PCR, where simple detection of an amplification product or estimation of the product's size is important. However, when high-fidelity DNA synthesis by PCR is required, such as gene cloning, or mutation analysis, the *Pfu* DNA polymerase is used. *Pfu* is a proofreading DNA polymerase isolated from *Pyrococcus furiosus*, and exhibits the lowest error rate of any thermostable DNA polymerase studied. Successful PCR using *Pfu* is readily performed requiring only slight modifications from PCR protocols optimized with *Taq*.

2.11.1. PCR working conditions

We used a thermal cycler, Minicycler PTC-100™ (M. J. Research), with Peltier refrigeration. The PCR reaction mix and the PCR conditions were the following:

PCR reaction mix	***Taq* polymerase**	***Pfu* polymerase**
10X PCR buffer	1X	1X
$MgCl_2$	1.5-2.5 mM	2 mM
Mixture of four dNTPs	0.20 mM	0.20 mM
Primer forward	50 pmol	50 pmol
Primer reverse	50 pmol	50 pmol
Template DNA	1 ng	10-50 ng
DNA polymerase	2.5 U *Taq*	2.5-5.0 U *Pfu*
bd. sterile H_2O up to	50 µl	50 µl

PCR conditions	***Taq* polymerase**		***Pfu* polymerase**	
First step	94 ºC	2-5 min	94 ºC	2-5 min
Denaturation	94 ºC	1 min	94 ºC	1min
Annealing	50-65 ºC	1 min	50-65 ºC	1 min
Polymerization	72 ºC	1 min/kb	72 ºC	2 min/kb
25-35 cycles from step 2				
Final extension	72 ºC	10 min	72 ºC	10 min

At lower annealing temperatures the amplification is more efficient, but the amount of mispriming is significantly increased. At higher temperatures (50-65 ºC), the specificity of the amplification reaction is increased, but its overall efficiency is reduced. Usually, the annealing temperature chosen is 2 ºC less than the lower melting temperature (T_m) of the two primers used. The PCR products are analyzed by electrophoresis in agarose gels.

2.11.2. Primer design

Some primer design considerations have to be borne in mind for PCR and sequencing experiments.

The length of the oligonucleotide will be 18 plus one extra base for each 2% decrease related to the 50% of G + C. High A + T percentages prevent the correct annealing of the primer, while high G + C percentages could produce unspecific amplifications due to accidental homology with G +C regions of the template DNA. 40-60% is the ideal range of G + C. Finally, a G + C equilibrated composition at the 3' terminus of the primer is recommended because this is where the amplification starts.

2.11.3. PCR-Preps

To select the desired recombinant DNA among the bacterial colonies obtained after DNA transformation, a rapid analysis by PCR could be used. A PCR reaction is performed for each isolated bacterial colony by touching one by one with a sterile tip first the colony, then a new LB plate with the appropriate antibiotic and then the PCR mixture placed in a PCR tube. A few cells are enough for the PCR reaction to amplify the desired DNA. The first step of the PCR is 5 min at 94 ºC, to break the bacterial membrane by heating and release the internal DNA. The PCR products are analyzed by electrophoresis in agarose gels.

2.12. DNA SEQUENCING

For DNA sequencing we used the *BigDye Terminator v3.1 Cycle Sequencing Kit* (Applied Biosystems, Ref. 4337455) following the manufacturer's instructions. This kit contains fluorescent nucleosides therefore sequencing products can be detected by fluorescence. The sequence of a single or double-stranded DNA can easily be determined with this kit using an oligonucleotide and performing a PCR reaction. The PCR sequencing mix and the PCR conditions are the following:

PCR sequencing mix	
Template double-stranded DNA (dsDNA)	200-500 ng
PCR product DNA	1-100 ng
Primer	3.2 pmol
Ready reaction mix	2 μl
bd. sterile H_2O up to	10 μl

PCR conditions		
First step	96 ºC	1 min
Denaturation	96 ºC	10 sec
Annealing	50 ºC	5 sec
Polymerization	60 ºC	4 min
Cycles from step 2	24	

The primer must anneal near the DNA fragment to be sequenced because the PCR reaction extendeds up to 700 bp away from the primer annealing site. After the PCR amplification, samples are precipitated with ethanol for 15 min at r.t., centrifuged at 16,000 x *g* for 20 min in a microcentrifuge and washed 3 times in 70% ethanol. Once samples are air-dried (protected from the light) they can be stored at -20 ºC.

The polyacrylamide-urea gel electrophoresis and the later analysis of the samples in an *ABI PRISM 3700 DNA Analyzer* (Applied Biosystems) were performed at the Scientific-Technique Services of the University of Barcelona.

2.13. RT-PCR

RT-PCR (reverse transcriptase-polymerase chain reaction) consists of three steps. In the first step, the contaminating genomic DNA is eliminated. The second step is the reverse transcription (RT) reaction (also called first strand synthesis of cDNA), a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA). Finally, in the third step the resulting cDNA is amplified by PCR.

2.13.1. Elimination of contaminating DNA

To prevent amplification of any contaminating DNA present in the RNA solution, RNA is treated with DNase I (RNase-free) from Roche (Ref. 10 776 785 001). DNase I is a nonspecific endonuclease that degrades double-and single-stranded DNA and is frequently used to remove contaminating DNA in RNA preparations. To preserve RNA integrity, a RNase inhibitor (RNase Out; from Invitrogen, ref. 10777-019) is included in the reaction.

Reaction components	
RNA	5-10 µg
1M Tris-HCl pH 7.8	1 µl
50 mM $MgCl_2$	4 µl
RNase Out 40 U/µl	1 µl
DNase I 10 U/µl	2 µl
DEPC H_2O up to	100 µl

The reaction is conducted for 90 minutes at 37 ºC. After this time, DNase I is inactivated by heat treatment for 10 min at 75 ºC.

2.13.2. Reverse transcription

This technique allows the synthesis of cDNA from a single-stranded RNA template. The polymerization reaction is catalysed by a RNA-dependent DNA polymerase (reverse transcriptase). In our case, the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) was used for the synthesis of cDNA (Invitrogen, Ref. 28025-013).

Reverse transcriptases require a primer to initiate synthesis of DNA. For the cloning of cDNAs, the most frequently used primer is oligo(dT) 12-18 nucleotides in length, which binds to the poly(A) tract at the 3´ terminus of eukaryotic mRNA molecules. The primer is added to the reaction mixture in large molar excess so that each molecule of mRNA binds several molecules of oligo(dT). Priming of cDNA synthesis begins from the most proximal of these bound primers and is very efficient.

Prior to the reaction, RNA is heated for 2 min at 85 ºC to denature RNA secondary structure and then, is kept on ice.

The components of the reaction mix are:

Reverse transcription	
5 X M-MLV RT buffer	4 µl
100 mM DTT	2 µl
RNase Out 40 U/µl	1 µl
2 mM dNTPS	5 µl
Oligo(dT) (500 ng/µl)	1 µl
RNA	4 µg
M-MLV RT 200 U/µl	1 µl
DEPC H_2O up to	20 µl

The reaction is incubated for 90 min at 37 ºC, and after this time, the reverse transcriptase is inactivated by heating at 70 ºC for 15 minutes.

The cDNA can now be used as a template for amplification by PCR (Section 2.11). For the PCR reaction, 2 µl of cDNA were used.

2.14. PCR-BASED MUTAGENESIS METHOD

The PCR technique was used for the introduction of mutations in a DNA sequence. The *QuikChange*™ *Site-Directed Mutagenesis Kit* (Stratagene, Ref. 200518) is used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids. The basic procedure utilizes a supercoiled dsDNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *Pfu* Turbo DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks.

Following PCR, the product is treated with *Dpn* I (10 U). The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I

digestion. The nicked vector DNA containing the desired mutations is then transformed into DH5α competent cells that will repair the nicks in the mutated plasmid.

Then, four of the transformed cells are cultured in LB with the appropriate antibiotic and plasmid DNA is obtained. Finally, the appropriate substitutions, as well as the absence of unwanted mutations, are confirmed by sequencing the inserts.

The following considerations should be made for designing mutagenic primers:

a) Both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
b) Primers should be between 25 and 45 bases in length, with a T_m of ≥ 78°C.
c) The desired mutation should be in the middle of the primer with 10–15 bases of correct sequence on both sides.
d) The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

PCR conditions		
First step	95 ºC	30 sec
Denaturation	95 ºC	30 sec
Annealing	55 ºC	1 min
Polymerization	68 ºC	2 min/kb
Cycles from step 2	18	

PCR reaction mix	
10X Reaction buffer (contains 20 mM $MgSO_4$)	1X (2 mM $MgSO_4$)
Mixture of four dNTPs	0.20 mM
Primer forward	125 ng
Primer reverse	125 ng
Template DNA	5-50 ng
DNA polymerase	2.5 U *Pfu* Turbo
bd. sterile H_2O up to	50 µl

3. EXPRESSION AND PURIFICATION OF PROTEINS IN *E. coli*

For expression and purification of rat CrAT protein the GST Gene Fusion System (Amersham Biosciences) was used. The system consists of three major components: pGEX plasmid vectors (Section 1.4), a GST purification module, and a site-specific protease. GST fusion proteins are produced in *E.coli* cells containing a recombinant pGEX plasmid. Protein expression from a pGEX plasmid is under the control of the *tac* promoter, which is induced using the lactose analog isopropyl β-D-thiogalactoside (IPTG). Soluble fusion proteins are easily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B (Ref. 17-0756-01) with a batch method. Cleavage of the desired protein from GST is achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX plasmids. In our case, PreScission protease was used (Ref. 27-0843-01).

The following protocol is used for the expression and purification of GST fusion proteins:

1. Use a single colony of *E.coli* BL21 cells containing a recombinant pGEX plasmid to inoculate 40 ml of LB medium plus ampicillin (100 mg/L).
2. Incubate for 14 hours at 37 ºC with vigorous shaking.
3. Dilute the culture 1:100 into LB medium (500 ml) plus ampicillin, and grow to an OD_{600} of 0.6-0.8 (3-4 h) with vigorous agitation at 37 ºC.
4. Induce fusion protein expression by adding 200 mM IPTG to a final concentration of 0.1 mM and incubate for an additional 14 h at 18 ºC.
5. Harvest cells by centrifuging at 3000 x *g* for 10 min at 4 ºC.
6. Wash in 20 ml of ice-cold PBS. Cells could be stored at -80 ºC at this step.
7. Suspend the cell pellet by adding 50 µl of ice-cold PBS per ml of culture. Add 1 ml of a protease inhibitor cocktail for bacterial cells (Sigma, Ref. P 8465).
8. Disrupt cells by sonication in 6-8 ml aliquots on ice (three 45 sec bursts - amplitude 12 microns- at 15 sec intervals).
9. Centrifuge at 10,000 x *g* for 30 minutes at 4 ºC to remove cellular debris and transfer the supernatant to a 15 ml tube.

10. Add 1 ml of Glutathione Sepharose 4B, 50% slurry, equilibrated with PBS (500 µl bed volume is used).

11. Incubate with gentle agitation at 4 ºC for 1 h.

12. Centrifuge at 500 x *g* for 5 min at 4 ºC and discard the supernatant.

13. Wash the beads three times with 10 bed volumes of ice-cold PBS.

14. Wash again with 10 bed volumes of PreScission cleavage buffer.

15. For each ml of bed volume, mix 15 µl (30 U) of PreScission protease with 980 µl of PreScission cleavage buffer.

16. Rotate the suspension at 4 ºC o/n.

17. Centrifuge the suspension at 500 x *g* for 5 min at 4 ºC and collect the eluate that contains the protein of interest.

18. Check the purity of the eluted protein by SDS-PAGE (Section 6.2.1.)

200 mM IPTG: Dissolve 477 mg IPTG into 10 ml of bd.H_2O. Filter through a 0.22 µm disposable filter.

PBS: 8 g/L NaCl, 1.44 g/L Na_2HPO_4, 0.2 g /L KCl, 0.24 g/L of KH_2PO_4 dissolved in 1 L of bd. water. Then, pH is adjusted to 7.4. Store at r.t.

PreScission cleavage buffer	
Tris-HCl pH 7.0	50 mM
NaCl	150 mM
EDTA	1 mM
DTT	1 mM

4. EXPRESSION OF PROTEINS IN YEAST

4.1. GROWTH OF *SACCHAROMYCES CEREVISIAE* STRAINS

S. cerevisiae grows well at 30 ºC with good aeration and agitation, and glucose as a carbon source. A rich medium, YPD, was used for growing *S. cerevisiae*. The density of the cells in a culture was determined spectrophotometrically by measuring its optical density (OD) at 600 nm. An OD_{600} of 1 is equal to ~3 x 10^7 cells/ml.

YPD medium: 10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose dissolved in distilled water. The pH was adjusted to 6.6 before autoclaving. The medium is kept at r.t.

Solid YPD medium: YPD medium plus 2% (w/v) agar. The pH was adjusted to 7.2 before autoclaving. When the temperature is about 50 ºC, the medium is distributed into 10-cm plates with 30 ml of medium each. Plates are stored at 4 ºC.

4.2. TRANSFORMATION OF YEAST CELLS

The lithium acetate procedure was used for yeast transformation (Schiestl, 1989). This method is based on the fact that alkali cations make yeast competent to take up DNA. After yeast is briefly incubated in buffered lithium acetate, transforming DNA is introduced with carrier DNA. Addition of polyethylene glycol (PEG) and a heat shock trigger DNA uptake. The yeast are then plated on selective media.

1. The night before the transformation, inoculate 10 ml YPD medium with a single yeast colony of the strain to be transformed (or 10 µl from a liquid culture). Grow o/n to saturation at 30 ºC.
2. Inoculate 50 ml YPD medium with preculture to an OD_{600} between 0.2 and 0.3 and grow for 3 h at 30 ºC.
3. Harvest cells by centrifuging 5 min at 100 x *g*, r.t.
4. Wash cells in 30 ml of sterile milliQ water and centrifuge 5 min at 100 x *g*.
5. Resuspend in 200 µl of ice cold buffered lithium solution (1X TE /1X lithium acetate) and keep on ice.

6. For each transformation, mix 100 µg carrier DNA with 100 ng transforming DNA.

7. Add 100 µl competent yeast suspension and mix thoroughly and keep on ice.

8. Add 600 µl PEG solution (1X TE/1X Lithium acetate/PEG 40%) and shake 30 min at 30 ºC.

9. Add 70 µl DMSO and heat shock 15 min at 42 ºC. Centrifuge 5 sec at r.t.

10. Resuspend yeast in 100 µl 1X TE buffer and spread up onto complete minimal (CM) medium plates selective for the plasmid marker.

11. Incubate at 30 ºC until transformants appear (2 to 3 days).

Solutions:

10X lithium acetate stock solution: 1 M lithium acetate, pH 7.5, filter sterilized.
10X TE buffer, pH 7.5, filter sterilized.
50% (w/v) PEG 4000, filter sterilized.
Carrier DNA: DNA (type III, sodium salt from salmon testes).

4.3. SELECTION AND GROWTH OF THE TRANSFORMANTS

Only the yeast cells that have incorporated the transforming DNA (yeast expression plasmid) are able to grow on plates lacking a specific amino acid. Positive transformants containing the pYES plasmid are selected on CM minus uracil (CM^{-Ura}) plates, whereas cells that have incorporated the pHVX2 plasmid are selected on CM minus leucine (CM^{-Leu}) plates.

Positive *S. cerevisiae* colonies are then inoculated into 30 ml of CM^{-Ura} or CM^{-Leu} medium with 2% glucose as carbon source and grown to an OD_{600} ~ 1. This liquid stock culture can be kept at 4 ºC for up to one month.

Then, the transformant growth is different depending on the expressed protein. When rat CrAT and L-CPT I is being expressed, to increase the number of mitochondria in the cell, yeast cells are grown in lactic acid. An appropriate amount of the glucose culture is inoculated in CM^{-Ura} plus 2% lactic acid (so that the OD_{600} = 0.1), and grown

at 30 ºC with vigorous agitation to an OD_{600} of 1. To induce protein expression, 2% of galactose is added and growth is continued for 20 h at 30 ºC.

For rat COT expression, is not necessary to grow the cells in lactic acid. An appropriate amount of the stock culture is inoculated in CM^{-Ura} plus 2% glucose and grown at 30 ºC with vigorous agitation to an OD_{600} of 1. Then, cells are harvested and resuspended in CM^{-Ura} plus 2% galactose and growth is continued for 20 h at 30 ºC.

Finally, when yeast CrAT is being expressed an appropriate amount of the stock culture is inoculated in CM^{-Leu} plus 2% glucose and grown at 30 ºC with vigorous agitation to an OD_{600} of 1.

CM dropout medium: 5 g/L $(NH_4)_2SO_4$, 1.7 g/L yeast nitrogen base (YNB) without ammonium sulphate, and 0.77 g/L of yeast dropout powder without uracil (CM^{-Ura}) or leucine (CM^{-Leu}). The carbon source, always used at a concentration of 2% w/v, can be glucose, galactose or lactic acid. The pH was adjusted to 5 before autoclaving.

Solid CM dropout medium: CM dropout medium plus 2% (w/v) agar. It is autoclaved immediately. The pH was adjusted to 6 before autoclaving. When the temperature is about 50 ºC, the medium is distributed into 10-cm plates with 30 ml of medium each. Plates are stored at 4 ºC.

4.4. PREPARATION OF PROTEIN EXTRACTS FROM YEAST

The glass beads procedure was used for preparing protein extracts. In this method, cells are disrupted by vigorous vortexing in the presence of glass beads.

4.4.1. Crude extracts

For the production of crude extracts the following protocol was used:

1. Harvest cells by centrifuging 5 min at 3,000 x *g* at 4 ºC.
2. Wash cells in 20 ml ice-cold distilled water.
3. Resuspend in buffer A with protease inhibitors.
4. Add the same volume of cold, acid-washed glass beads.

5. **5.** Disrupt cells by vigorous vortexing: four pulses of 1 min, samples are kept on ice for 1 min after each pulse.
6. **6.** Centrifuge homogenates at 500 x *g* for 5 min at 4 ºC, to remove cell debris and glass beads.
7. **7.** Crude extract is further centrifuged at 11,000 x *g* for 10 min at 4 ºC.
8. **8.** Resuspend pellet in buffer A with protease inhibitors.
9. **9.** Freeze and store the samples at -80 ºC.

Buffer A: 150 mM KCl, 5 mM Tris-HCl pH 7.2

Protease inhibitors: 1 mM PMSF, 0.5 μM benzamidine, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A.

4.4.2. Mitochondrial fraction

For the isolation of the mitochondrial fraction from yeast the following protocol was used:

1. **1.** Harvest cell by centrifuging 5 min at 3,000 x *g* at 4 ºC.
2. **2.** Wash twice in 20 ml of ice-cold distilled water.
3. **3.** Resuspend in buffer B with protease inhibitors.
4. **4.** Add the same volume of cold, acid-washed glass beads.
5. **5.** Disrupt cells by vigorous vortexing: ten pulses of 30 sec, samples are kept on ice for 30 sec after each pulse.
6. **6.** Centrifuge homogenates at 500 x *g* for 10 min at 4 ºC.
7. **7.** Collect supernatant and centrifuge at 17,000 x *g* for 30 min at 4 ºC.
8. **8.** Resuspend the pellet (mitochondria) in buffer B with protease inhibitors.
9. **9.** Freeze and store the mitochondrial fraction at -80 ºC.

Buffer B: 10 mM HEPES pH 7.8, 1 mM EDTA, 10% glycerol

Protease inhibitors: 1 mM PMSF, 0.5 μM benzamidine, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A.

5. FERMENTATION TRIALS WITH YEAST

5.1. FERMENTATION CONDITIONS

S. cerevisiae FY23 strains, containing the pHVX2 vector alone or different pHVX2 constructs, were used for the fermentation experiments. Triplicate starter cultures were produced for these yeasts by growing cells in a minimal synthetic dextrose (SD) medium lacking leucine (SD^{-Leu}) for ~24 hours to stationary phase at 28 °C at 200 rpm. The starter cultures were inoculated in equal cell number at a ~100-fold dilution into 150 ml of a synthetic fermentation medium supplemented with the appropriate amino acids and L-carnitine.

Fermentations were conducted at 28 ºC in 250 mL conical flasks fitted with an air lock filled with sterile water and side arm septa for sampling. The fermentations were not agitated. Samples for chromatographic analysis (50 ml) were taken after 4 days and 7 days of fermentation, clarified by centrifugation (2,000 x *g* for 5 min) to remove yeast cells, and supernatants were stored at -20 ºC until further analysis. Fermentations were performed in triplicate.

SD^{-Leu} medium: 6.7 g/L YNB without amino acids, and 20 g/L glucose (2%). The medium is supplemented with 20 mg/L of uracil and 30 mg/L of tryptophan. The pH was adjusted to 5 before autoclaving.

Fermentation medium (SD^{-Leu}): 6.7 g/L YNB without amino acids, and 80 g/L glucose (8%). The medium is supplemented with 20 mg/L of uracil and 30 mg/L of tryptophan and 100 mg/L of L-carnitine. The pH was adjusted to 5 before autoclaving. L-carnitine was added alter autoclaving**.**

5.2. ANALYSIS OF FERMENTATION PRODUCTS

The quantification of the fermentation products was carried out using gas chromatography/mass spectrometry/headspace-solid phase microextracion/stable isotope dilution analysis (HS-SPME-SIDA-GC/MS) with deuterium labelled analogues

as the internal standards (Siebert, 2005). A total of 31 volatile fermentation-derived products that contribute to the aroma of wine were quantified, including volatile ethylesters, acetates, alcohols and acids. Table 1 lists a range of important fermentation-derived volatile ethyl esters, acetates, acids and alcohols.

Analyte	Aroma descriptors	Calibration range (μg/l) (1/10 dilution)[a]	Calibration equation	R^2
Ethyl acetate	VA, nail polish	1–5,000	$y = 1.1231x$	0.9999
Ethyl lactate	Strawberry	50–5,000	$y = 0.0121x - 0.00125$	0.9990
Ethyl propanoate	Fruity	0.1–500	$y = 0.751x$	1.0000
Ethyl 2-methylpropanoate	Fruity	0.1–500	$y = 0.7738x$	0.9999
Ethyl butanoate	Acid fruit	0.1–500	$y = 0.9067x + 0.0001$	1.0000
Ethyl 2-methylbutanoate	Sweet fruit	0.1–500	$y = 0.7988x + 0.0002$	0.9991
Ethyl 3-methylbutanoate	Berry	0.2–500	$y = 0.1269x + 0.0001$	0.9986
Ethyl hexanoate	Green apple	0.5–200	$y = 0.0989x + 0.0001$	0.9994
Ethyl octanoate	Sweet, soap	0.2–100	$y = 0.8662x + 0.0004$	0.9996
Ethyl decanoate	Pleasant, soap	0.1–200	$y = 0.5257x + 0.0013$	0.9974
Ethyl dodecanoate	Soapy, estery	0.1–200	$y = 0.4197x + 0.0004$	0.9994
2-Methylpropyl acetate	Banana, fruity	0.1–500	$y = 4.0557x + 0.0106$	1.0000
2-Methylbutyl acetate	Banana, fruity	0.5–500	$y = 0.4075x + 0.0013$	0.9999
3-Methylbutyl acetate	Banana	2.0–200	$y = 0.8937x + 0.0049$	0.9998
Hexyl acetate	Sweet, perfume	0.1–100	$y = 1.1814x + 0.0007$	0.9999
2-Phenylethyl acetate	Flowery	0.5–100	$y = 4.9794x + 0.0079$	0.9998
2-Methylpropanol	Fusel, spiritous	5–5,000	$y = 2.7254x + 0.0022$	0.9997
Butanol	Fusel, spiritous	25–25,000	$y = 0.9521x - 0.0003$	0.9996
2-Methylbutanol	Nail polish	250–10,000	$y = 2.5049x + 0.0283$	0.9998
3-Methylbutanol	Harsh, nail polish	250–10,000	$y = 1.2226x + 0.0095$	0.9984
Hexanol	Green, grass	0–500	$y = 1.2386 - 0.0002$	0.9999
2-Phenylethanol	Roses	25–2,500	$y = 2.0863x + 0.0019$	0.9997
Acetic acid	VA, vinegar	200–20,000	$y = 0.9465x + 0.0203$	0.9993
Propanoic acid	Vinegar	20–500	$y = 0.1793x^2 + 0.5423x + 0.0824$	0.9944
2-Methylpropanoic acid	Cheese, rancid	10–2,000	$y = 0.1483x^2 + 0.8125x - 0.0046$	0.9999
Butanoic acid	Cheese, rancid	5–500	$y = 0.1488x^2 + 1.3391x + 0.0919$	0.9994
2-Methylbutanoic acid	Cheese, sweaty	5–500	$y = 0.2685x^2 + 2.0783x - 0.06$	0.9996
3-Methylbutanoic acid	Blue cheese	5–500	$y = 0.1788x^2 + 3.2539x + 0.0078$	0.9949
Hexanoic acid	Cheese, sweaty	5–500	$y = 0.2518x^2 + 1.5596x + 0.0359$	0.9998
Octanoic acid	Rancid, harsh	10–1,000	$y = 0.5206x^2 + 0.7157x - 0.0044$	0.9995
Decanoic acid	Fatty	5–200	$y = 0.6512x^2 + 1.0737x - 0.0136$	0.9985

Table 1. **Aroma descriptors, calibration ranges, calibration equations and R^2 values for various fermentation-derived compounds.**

The samples were analysed by GC/MS using an Agilent 6890 gas chromatograph equipped with Gerstel MPS2 multi purpose sampler and coupled to an Agilent 5973N mass selective detector. The instrument was controlled and the data analysed with G1701CA ChemStation software. The gas chromatograph was fitted with a 60 m x 0.25 mm Phenomenex fused silica capillary column ZB-Wax, 0.25 μm film thickness. The carrier gas was helium (Ultra High Purity), linear velocity 36 cm/s, flow rate 2 ml/min in constant flow mode. The oven temperature was started at 40 ºC, held at this temperature for 4 min, then increased to 220 ºC and held at this temperature for 20 min. The inlet was fitted with a SPME inlet liner and held at 200 ºC. A headspace solid phase microextraction (SPME) of the sample was injected in the pulsed splitless mode and the splitter, at 25:1, was open after 30 sec. The mass spectrometer quadrupole temperature

was set at 106 ºC, the source was set at 230 ºC and the transfer line is held at 250 ºC. Positive ion electron impact spectra at 70eV are recorded in selective ion monitoring mode.

The conditions for headspace SPME sampling were as follows: a 10 ml aliquot of diluted sample (1 in 10 dilution in Milli-Q water) was added to a 20 ml vial containing 2 g of NaCl, and the vial was immediately crimp-capped. Subsequently, 100 μl of combined internal standards solution was injected through the septum and the vial was shaken well. The vial and its contents were heated to 35 ºC. The Supelco Carbowax/divinylbenzene (CW/DVB) 65 μm fibre was exposed to the headspace for 10 min.

The accuracy of the analysis was achieved with the use of poly-deuterated internal standards for stable isotope dilution analysis (SIDA). Confirmation of identity was achieved by retention time and peak shape, and measurement of at least three ions for each analyte and internal standard with the MS operating in selected ion monitoring mode to facilitate more precise quantification with a high sampling rate.

6. PROTEIN ANALYSIS

6.1. BRADFORD PROTEIN QUANTIFICATION

The protein determination method (Bradford, 1976) is based on the fact that some colorants, when adsorbing onto protein molecules, change their absorption spectrum. The Bradford reagent contains copper in an acid medium of orthophosphoric acid and methanol. When the protein binds to copper its absorption maximum shifts in an acidic solution from 465 to 595 nm. This method, which has little interference, has large sensitivity and linearity.

Protein is measured according to the manufacturer's instructions (BioRad protein assay, ref. 500-0006), using bovine serum albumin (BSA) as a protein standard in the range of 2-20 μg/ml. Bradford reagent is diluted 1/5 in water. BSA standard stock is prepared in water at 1 μg/μl and the absorbance of the blank, standard curve or the samples is measured at 595 nm in a spectrophotometer with plastic cuvettes in a total volume of 1 ml.

6.2. WESTERN-BLOT

The Western-blot technique detects, with a specific antibody, a protein between a sample of proteins that have been separated by electrophoresis and transferred to a nitrocellulose membrane. Western-blot was applied for the analysis of CrAT and COT proteins. The procedure has the following steps: electrophoresis, transference, antibody incubation (Western-blot) and detection.

6.2.1. Electrophoresis

Electrophoresis in polyacrylamide-SDS gels (SDS-PAGE) is the most widely used technique for resolving proteins denatured with SDS (Laemmli, 1970). Proteins and protein subunits are separated by its MW when they are migrating through the gel towards the anode.

Sample preparation: *S. cerevisiae* protein extracts expressing CrAT (8 μg of the mitochondrial fraction) or COT (10 μg of the crude extract) were diluted in 4X loading buffer. Samples are heated at 95 ºC for 5 min. Then, they are briefly centrifuged and placed on ice before loading on-to the gel.

Gel preparation: For CrAT and COT proteins, an 8% separating gel is prepared. Separating gel is prepared by adding the polymerizing agent TEMED at the end and it is immediately cast between the two glasses of the electrophoresis apparatus. Some drops of isopropanol are added on the top to achieve a straight edge of the gel. Once the gel is polymerized isopropanol is removed. Then, a 5% stacking gel is prepared and cast and a 1.0 mm comb is adjusted between the two glasses. Both the separating and stacking gel take approximately 20 min to polymerize.

Electrophoresis performance: To run the electrophoresis, the comb is removed and electrophoresis buffer is added on to the electrophoresis chamber and on to the sample loading wells. Samples and protein marker (Invitrogen, ref. LC5925) are loaded on to the gel and electrophoresis is performed at a constant current of 30 milliamps (mA) for 1 h approximately. Electrophoresis is stopped when bromophenol blue is just off the bottom of the gel.

4X Loading Buffer: 250 mM Tris-HCl pH 6.8, 8% SDS, 20% β-mercaptoethanol, 40% glycerol and 0.04% bromophenol blue. It is stored at -20 ºC.

4X Loading Buffer	
1.25 M Tris-HCl pH 6.8	5 ml
SDS	2 g
β-mercaptoethanol	5 ml
glycerol (87%)	11.6 ml
bromophenol blue	10 mg
H_2O up to	25 ml

SDS-PAGE recipe for one 1.0 mm gel:

	Separating gel (8%)	**Stacking gel (5%)**
bd.H_2O	4.4 ml	1.9 ml
40% Acrylamide/Bis mix	1.5 ml	312 µl
1.875 M Tris-HCl pH 8.8	1.5 ml	—
1.25 M Tris-HCl pH 6.8	—	250 µl
10% SDS	75 µl	25 µl
10% ammonium persulphate	25 µl	8.5 µl
TEMED	3.75 µl	2.5 µl
	Total volume = 7.5 ml	Total volume = 2.5 ml

Acrylamide is a potent cumulative neurotoxin. Contact with the skin and inhalation must be avoided. 10% ammonium persulphate is prepared by dissolving 0.5 g of ammonium persulfate in 5 ml of bd. H_2O and it is stored at -20 ºC.

1X Electrophoresis buffer: 25 mM Tris, 192 mM glycine and 0.1% SDS. It is stored at r.t. For 1 L:

Electrophoresis buffer	
Tris	3.03 g
Glycine	14.42 g
SDS	1 g
H_2O up to	1 L

6.2.2. Transference

Once electrophoresis is finished, stacking gel is removed and proteins in the separating gel are transferred to a nitrocellulose membrane for its later antibody incubation. The transference sandwich contains, in the following order from the negative to the positive pole: a sponge, 3 Whatman papers, the gel, the nitrocellulose membrane, 3 Whatman papers and another sponge. All must be submersed in transfer buffer avoiding bubbles. Whatman papers and the nitrocellulose membrane must have

the same size as the gel. The transference is performed at 4 ºC for 1 h at 250 mA. Once it is finished, membrane is washed in water and effectiveness of the transference is checked by Ponceau S solution (Sigma-Aldrich, ref. P-7170) staining of the protein bands. To remove the red staining, membrane is washed in PBS-Tween (PBS-T) for a few minutes.

1X Transfer buffer: 20 mM Tris, 20% methanol and 150 mM glycine.

Transfer buffer	
Tris	2.42 g
Glycine	11.2 g
Methanol	200 ml
H_2O up to	1 L

PBS-Tween: Tween 0.1% in 1X PBS. 1 ml of Tween-20 is diluted in 1000 ml of PBS. It is stored at r.t.

6.2.3. Antibody incubation

For the immunoblotting, we used the Enhanced ChemiFluorescence (ECF) Western Blotting kit (Amersham Biosciences, ref. RPN 5783-anti-rabbit), which allows the use of a Storm 840 Laser scanning system (Molecular dynamics, Amersham Biosciences) to quantify by fluorescence the intensity of the bands. This immunodetection permits the use of goat anti-rabbit alkaline phosphatase-linked immunoglobulin, followed by addition of ECF substrate. The alkaline phosphatase catalyses the conversion of ECF substrate to a highly fluorescent product which fluoresces strongly at 540-560 nm. According to the manufacturer's instructions the protocol is as follows:

Blocking the membrane: Non-specific binding sites are blocked by immersing the membrane in blocking solution for 1 h on an orbital shaker at r.t. After this time, membrane is rinsed and washed once for 15 min and twice for 5 min in PBS-T.

First antibody incubation: First antibodies used were the rabbit CrAT-specific polyclonal antibody against the full-length of rat CrAT (Cordente, 2004), and the rabbit

COT-specific antibody against amino acids 344-360 of rat COT (Caudevilla, 1998). First antibody is diluted (1/10,000 for CrAT and 1/100 for COT) in blocking solution. Incubation is done o/n at 4 ºC on an orbital shaker. After this time, membrane is rinsed and washed once for 15 min and twice for 5 min in PBS-T.

Second antibody incubation: Second antibody (anti-rabbit for rat CrAT and COT) is diluted 1/6,000 in PBS-T and it is incubated for 1 h at r.t. on an orbital shaker. Then, membrane is rinsed and washed once for 15 min and twice for 5 min in PBS-T.

Blocking solution: 5% (w/v) skimmed milk in PBS-T.

6.2.4. Detection

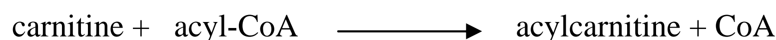
The membrane is incubated for 20 min with the ECF substrate. The volume of ECF should be enough to cover the membrane surface. After this time, the membrane is drained and placed directly on to the sample holder of the fluorescence scanning instrument (Storm 840, Molecular Dynamics) and intensity of the bands is quantified.

7. DETERMINATION OF ENZYMATIC ACTIVITY

Carnitine acyltransferase activity is determined by two different methods: a fluorometric assay for CrAT and COT proteins, and a radiometric method for inhibition assays and CPT I activity. Choline acetyltransferase activity is also determined by the fluorometric assay.

7.1. FLUOROMETRIC ASSAY

CrAT and COT activities were determined by an endpoint fluorometric method (Hassett, 2000) with minor modifications. The method measures the carnitine-dependent release of CoA from acyl-CoA according to the following reaction (forward reaction):



The CoA formed reacts with the thiol-reactive probe 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F) to give a highly fluorescent product enabling carnitine acyltransferase activity to be measured.

The assay is conducted for 8 min at 30 °C in a total volume of 600 µl. The final concentration of reaction components are:

Reaction mix	
HEPES pH 7.8	40 mM
EDTA	1.5 mM
*Acyl-CoA	0.1 mM
L-carnitine	1.5 mM

* Acetyl-CoA is used for CrAT and decanoyl-CoA for COT activity assays.

The procedure is as follows:

Samples were prepared in 1.5 ml tubes and reactions are started by the addition of 5 µg of yeast-expressed CrAT or COT, or from 0.1 to 5 µg of *E. coli*-expressed CrAT. Parallel (blank) assays were run in the absence of L-carnitine to determine carnitine-independent deesterification of fatty acyl groups from CoA (e.g., activity of acyl-CoA hydrolase). Reactions were arrested by heat treatment for 10 min at 70 ºC. Proteins were sedimented by centrifugation for 10 min at 13,400 x *g*; 550 µl of the supernatant was collected, 35 µl of 2 mg/ml solution of ABD-F was added and the mixture was incubated at 50 ºC for 30 min (ABD-F labelling of CoA is complete after this time).

Fluorescence intensities, indicative of the binding of the CoA thiol group to ABD-F, were measured in 10 mm path-length glass cuvettes (700 µl) at 391 nm (excitation wavelength) and 515 nm (emission wavelength). These fluorescence intensities were compared with a standard curve for CoA between 0-30 nmol. Standard curves for CoA/ABD-F were linear between this range. All fluorometer recordings were performed with a Perkin Elmer LS 45 luminescence spectrometer, and enzyme activities were measured in duplicate.

Specific activity (S.A.) of the enzyme is calculated as follows:

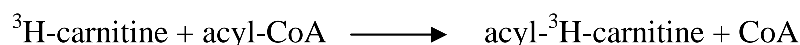
S.A. ($nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) = (F.I.) / (min x mg prot x slope)

Where F.I. is the fluorescence intensity, and "slope" is the slope of the CoA/ABD-F standard curve plot (approx. 12 fluorescence intensity units per nmol of CoA).
Values reported are the means ± S.D. of at least three independent determinations.

ABD-F stock solution: 50 mg of ABD-F is dissolved in 2.5 ml of DMSO to a concentration of 20 mg/ml. Aliquots are stored at -20 ºC. On the day of the experiment, the stock solution is diluted to 2 mg/ml in bd. water.
1 mM acyl-CoA: The stocks of 1mM acyl-CoA are prepared in 1 mM sodium acetate pH 5.9 and stored at -20 ºC.
50 mM L-carnitine: 10 mg of L-carnitine is dissolved in 10 ml of bd. water. L-carnitine is neutralized with KOH before use. Aliquots are stored at -20 ºC.

7.2. RADIOMETRIC ASSAY

Carnitine acyltransferase activity is also determined by a radiometric method as previously described (Morillas, 2000) with minor modifications. The substrates for the assay are L-[*methyl*-^{3}H]carnitine hydrochloride (Amersham Biosciences, ref. TRK762) and acyl-CoA and the reaction is done in the forward direction:



$$^{3}\text{H-carnitine} + \text{acyl-CoA} \longrightarrow \text{acyl-}^{3}\text{H-carnitine} + \text{CoA}$$

The procedure takes advantage of the fact that the product of the reaction, acyl-^{3}H-carnitine, is soluble in an organic medium. Therefore, it could be separated by extraction from the radioactive substrate, ^{3}H-carnitine, that has not been reacted and which will remain in the aqueous phase. This method is suitable for medium and long-chain acyl-CoAs but not for short-chain acyl-CoAs, since short-chain acylcarnitine esters are not soluble in an organic medium.

The final concentration of the components in the reaction are:

Reaction mix	
Tris-HCl pH 7.2	105 mM
KCl	15 mM
$MgCl_2$	4 mM
ATP	4 mM
GSH	250 µM
Palmitoyl-CoA	50 µM
L-[*methyl*-^{3}H]carnitine (0.3 µCi)	400 µM
defatted BSA	0.1%

The procedure is as follows:

The reaction mix is prepared in a 15 ml tube kept on ice. Samples are done in duplicate. Protein samples are prepared on ice in 1.5 ml tubes by diluting the protein in

4X reaction buffer and by adjusting the volume with bd. water up to 40 µl. The blank will contain bd. water instead of protein.

Protein samples	
Protein	5 µg
4X reaction buffer	10 µl
bd. water up to	40 µl

One by one, 160 µl of the reaction mix is added to each protein sample. Samples are vortex-mixed and placed on a water bath at 30 ºC for exactly 5 min. Reaction is stopped with the addition of 200 µl of 1.2 M HCl. Samples are vortex-mixed and extractions of the product of the reaction, acyl-^{3}H-carnitine, are done by adding 600 µl of water-saturated butanol. Samples are vortex-mixed and centrifuged for 2 min at 16,000 x *g* in a microcentrifuge. 400 µl of the upper phase is added to another 1.5 ml tube with 200 µl of bd. water. Samples are vortex-mixed and centrifuged for 2 min at 16,000 x *g*. 250 µl of the upper phase is counted in plastic scintillation vials with 5 ml of scintillation liquid (Ecolite, ICN). ^{3}H radioactivity is counted in a liquid scintillation analyzer (TRI-CARB 2100TR, Packard).

Specific activity (S.A.) of the enzyme is calculated as follows:

S.A.(nmol·min^{-1}·mg protein^{-1})= (cpm x 600 µl) / (S.R. x mg prot. x min x 250 µl)

Where cpm are the counts per minute and S.R. is the ^{3}H-carnitine specific radioactivity (approx. 3000 cpm/nmol).

<u>4X reaction buffer</u>: 420 mM Tris-HCl pH 7.2, 60 mM KCl, 16 mM $MgCl_2$.

<u>16 mM ^{3}H-carnitine</u>: 6.6 mg of cold carnitine is dissolved in 982.3 µl of 95% ethanol plus 982.3 µl of bd. water and 125 µl of L-[*methyl*-^{3}H]carnitine hydrochloride (80 Ci/mmol) is added. Aliquots are stored at -80 ºC.

7.2.1. Malonyl-CoA inhibition assay

For malonyl-CoA inhibition assays, the different amounts of malonyl-CoA are added to the protein samples. Samples are vortex-mixed and preincubated for 2 min at 30 ºC prior to the addition of the reaction mix. Carnitine acyltransferase activity assay is performed as described above.

2mM Malonyl-CoA: The stock of 2 mM malonyl-CoA is prepared in 1 mM sodium acetate pH 5.9 and stored at -20 ºC

7.2.2. Effect of C75-CoA on carnitine acyltransferase activity

7.2.2.1. Activation of C75 to C75-CoA

It has been described (Bentebibel, 2006) that C75 has to be previously activated to C75-CoA to interact with CPT I activity. This activation is done by a simple enzymatic method (Taylor, 1990) which uses the *Pseudomonas* sp. acyl-CoA synthetase (ACS).

The reaction is performed with 1 µmol of C75 (Alexis Biochemicals, ref. 270-286-M005) in a total volume of 1 ml of an activation buffer containing:

Activation buffer	
Triton X-100	0.1% (w/v)
CoA	5 mM
ATP	10 mM
DTT	1 mM
$MgCl_2$	10 mM
MOPS-NaOH pH 7.5	100 mM

The mix is sonicated for 5 min to permit C75 emulsion and 0.25 U of the acyl-CoA synthetase is added. The reaction is carried out at 35 ºC for 2 h and stock aliquots are stored at –80 ºC. It is assumed that all the C75 has been converted to C75-CoA and thus its final concentration in the solution is 1 mM. Excess CoA from the C75-CoA activation solution is removed by incubation for 30 min at 4 ºC with 1.25 ml of SulfoLink Coupling Gel (Pierce Biotechnology, Ref. 20401). Iodoacetyl groups on the Coupling Gel react specifically with the sulfhydryl group of CoA, and enables covalent

immobilization of the CoA molecule. After the incubation, the suspension is centrifuged for 5 min at 500 x *g* and the eluate is collected. The presence of residual CoA in the eluate was determined by the fluorometric method (section 7.1). SulfoLink Coupling Gel retains more than 95% of the initial CoA content.

7.2.2.2. C75-CoA inhibition assay

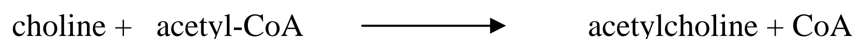
For C75-CoA inhibition assays, C75-CoA is added to the protein sample and preincubated for 5 min at 30 ºC, as described for the malonyl-CoA inhibition assay. Then, protein samples are vortex-mixed and carnitine acyltransferase activity assay is performed as described in Section 7.2. C75-CoA concentrations ranging from 1 to 60 µM were used to estimate the IC_{50} value. IC_{50} corresponds to the inhibitor concentration that inhibits 50% of the enzyme activity. Inhibition constants (K_i and K_{inact}) were determined at 50 µM acyl-CoA concentration by nonlinear parameter estimation (Maurer, 2000), using the following equation and SigmaPlot software.

$$\ln (E/E_o) / t = - K_{inact} \bullet [I] / ([I] + K_i)$$

Where E_o represents the maximal enzyme activity following preincubation in the absence of inhibitor (I), E represents the activity following preincubation with inhibitor, and t represents the inhibitor preincubation time.

7.3. DETERMINATION OF CHOLINE ACETYLTRANSFERASE ACTIVITY

Choline acetyltransferase activity is measured by the same fluorometric method described above (Section 7.1.). The substrates for the assay are acetyl-CoA and choline. The reaction is done in the following direction:



The assay was conducted for 8 min at 30 °C in a total volume of 600 µl. The final concentration of reaction components are:

Reaction mix	
HEPES pH 7.8	40 mM
EDTA	1.5 mM
Acetyl-CoA	0.1 mM
Choline	20 mM

7.4. KINETIC PARAMETERS

For determination of the K_m for acyl-CoA in the presence of carnitine as the acceptor of the acyl group, carnitine concentration was fixed at 1.5 mM (unless otherwise indicated) and acyl-CoA concentrations ranged from 2 to 150 µM.

For determination of the K_m for acyl-CoA in the presence of choline as the acceptor of the acyl group, choline concentration was fixed at 100 mM and acyl-CoA concentrations ranged from 2 to 150 µM.

For determination of the K_m for carnitine, acyl-CoA was fixed at 0.1 mM and carnitine concentrations ranged from 20 to 1600 µM.

For determination of the K_m for choline, acyl-CoA was fixed at 0.1 mM and choline concentrations ranged from 2 to 300 mM.

K_m and V_{max} values were determined by fitting the data using non-linear regression analysis to the Michaelis-Menten equation with SigmaPlot software. Catalytic efficiency was defined as V_{max} /K_m for yeast-expressed CrAT, and as K_{cat} /K_m for *E.coli*-expressed CrAT.

8. BIOINFORMATIC METHODS

8.1. BASIC BIOINFORMATIC TOOLS AND DATABASES

Protein sequences were obtained from the Swiss Prot data bank (http://www.expasy.org/sprot/) and DNA sequences from the GenBank Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Yeast protein and DNA sequences were obtained from the Saccharomyces genome database (http://www.yeastgenome.org/).

The RCSB Protein Data Bank (RSCB-PDB, http://www.rcsb.org/pdb/) is a repository for the processing and distribution of 3-D biological macromolecular structure data. This website was used to obtain the currently available crystallographic data structures of proteins of the carnitine/choline acyltransferase family.

RasMol (version 2.7.1., http://www.RasMol.org) was used for the visualisation of proteins, nucleic acids and small molecules. The program reads in a molecule coordinate file and interactively displays the molecule on the screen in a variety of colour schemes and molecule representations.

Restriction maps of the genes of interest were obtained from the Gene/Sequence resources page of the Saccharomyces genome database (http://db.yeastgenome.org/cgi-bin/seqTools).

The Basic Local Alignment Search Tool (BLAST) (Altschul, 1990; Altschul, 1997) was used to identify sequences of members of the carnitine/choline acyltransferase family. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches (http://www.ncbi.nlm.nih.gov/BLAST/).

The EMBOSS-Transeq tool, (European Bioinformatic Institute (EBI) website, http://www.ebi.ac.uk/emboss/transeq/) was used for the translation of a nucleic acid sequence to the corresponding amino acid sequence.

The BRENDA database (http://www.brenda.uni-koeln.de) is a resource for data of enzymes. This database was used to obtain information of the enzymes of the carnitine/choline acyltransferase family, including data on the occurrence, catalyzed reaction, kinetics, substrates/products, inhibitors, structure and stability.

8.2. SEQUENCES ALIGNMENTS AND COMPARISONS

Multiple alignment of nucleic acids or protein sequences was performed using the program ClustalW (Thompson, 1994) that can be found at the EBI website (http://www.ebi.ac.uk/clustalw/index.html).

The EMBOSS-Align tool (EBI website, http://www.ebi.ac.uk/emboss/align/) is used for pairwise alignment of sequences. This program compares two sequences and determines the percent identity and similarity between them.

8.3. SUBFAMILY CONSERVED RESIDUES ANALYSIS

The analysis of differentially conserved residues (tree determinants) among short-chain acyltransferases (ChAT and CrAT), medium-chain acyltransferases (COT) and long-chain acyltransferases (CPTs) was done with the SequenceSpace algorithm (Casari, 1995; del Sol Mesa, 2003), using the multiple sequence alignment of the carnitine/choline acyltransferase family of proteins as input.

8.4. CONSTRUCTION OF THE CrAT, COT AND CPT I MODELS

The structural models for wt CrAT and wt COT were constructed using homology modelling procedures based on the multiple, structure-based alignment of the rat CrAT and COT amino acid sequences with members of the carnitine acyltransferase family, including the 3-D structures of human CrAT (PDB entry 1NM8); mouse CrAT (free enzyme structure: PDB accession number 1NDB; carnitine complex: 1NDF; CoA complex: 1NDI); and mouse COT (free enzyme structure: 1XL7; octanoylcarnitine complex: 1XL8). Liver CPT I (L-CPT I) structural model was constructed using as a template the structure of mouse COT (1XL7, 1XL8).

The 3-D models were built using the program Swiss-Pdb Viewer and the SWISS-MODEL server facilities (Guex, 1997; Guex, 1999; Peitsch, 1995; Peitsch, 1996) (http://www.expasy.ch/swissmod/SWISS-MODEL.html).

The structural quality was checked using the WHAT-CHECK routines (Hooft, 1996) from the WHAT IF program (Vriend, 1990) and the PROCHECK validation program from the SWISS-MODEL server facilities (Laskowski, 1993).

The 3-D models of CrAT mutants M564G, M564A, D356A/M564G, T465V/T467N/R518N and A106M/T465V/T467N/R518N, and COT mutant G553M were built by the same procedures using the structures obtained for the respective wt enzymes as templates.

Finally, 3-D coordinates of rat ChAT structure were obtained from the PDB entries 1Q6X and 1T1U.

8.5. MOLECULAR DOCKING

Docking calculations to obtain a molecular model of the interaction between the substrates acetyl-, decanoyl-, myristoyl-, palmitoyl-, stearoyl-, and arachidoyl-CoA and the 3-D models of the putative receptor proteins wt CrAT, wt COT, wt L-CPT I, CrAT M564G, CrAT D356A/M564G and COT G553M were performed using the programs Autodock (Goodsell, 1996; Morris, 1998) and Hex (Ritchie, 2000). Protein targets and ligands (acyl-CoAs and carnitine) were prepared using the algorithms Addsol and Autotors from the Autodock package. To ensure a complete search of binding sites available for acyl-CoAs independent docking calculations were performed. In order to sample their conformational space intensively we used the whole set of rotatable bonds in the acyl chains of the ligands. Only docking models with their CoA residue positions close to those found in the 1NDI crystal were considered for further steps. Finally, among the position clusters selected for each ligand, the model with the lowest docking energy for each particular interaction was considered.

The structural interactions between the substrates choline and carnitine and the rat wt ChAT, wt CrAT and CrAT mutant proteins T465V/T467N/R518N and

A106M/T465V/T467N/R518N, respectively, were performed using the methods implemented in the Autodock suite. In order to preserve the structural position and conformation of carnitine and choline respectively within the active sites indicated in the original publications of CrAT and ChAT structures, rigid docking models were obtained for every putative interaction. Only docked models locating the common trimethylammonium group of both choline and carnitine in the originally suggested sites were considered for refining. As in the case of the acyl-CoA substrates, the lowest energy docked models were selected within the filtered sets.

9. STATISTICAL ANALYSIS

Data are expressed as means ± S.D. for at least three independent experiments performed in duplicate. Different experimental groups were compared with the Student's *t* test. A probability level of $p<0.05$ was considered to be statistically significant.