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L-Carnitine via Enzyme-Catalyzed Oxidative Kinetic Resolution†

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Abstract—L-Carnitine of high optical purity was prepared via kinetic resolution using a mutant strain of *Acinetobacter calcoaceticus* ATCC 39647. This organism preferentially metabolized the D-enantiomer of the racemate to furnish L-carnitine. Recovery of L-carnitine was 93 %, providing a total weight yield of 46.5 % in 92 % enantiomeric excess. The mode of degradation of carnitine was shown to proceed via a monooxygenase-catalyzed oxidative cleavage resulting in the formation of trimethylamine and malic acid. The data suggest that the stereoselective metabolism of DL-carnitine is probably the result of differential permeability of the cell membrane towards the optical antipodes.

Introduction

L-Carnitine was isolated from meat[1] in 1905 but its important role as a carrier of medium and long chain fatty acids across the inner mitochondrial membrane was not known until 1955.[2] Since 1973, an increasing number of human carnitine deficiency cases has been recognized and successfully treated by replacement therapy[3] and in 1984 L-carnitine acquired orphan drug status in the U.S. Moreover, some apparent beneficial effects have been observed upon the oral administration of L-acetylcarnitine to neuropathic patients.[4] Because D-carnitine is a competitive inhibitor of L-carnitine acyl transferases[5] and can deplete the L-carnitine level of heart tissues, the availability of enantiomerically-pure L-carnitine is vital for human therapy.

Consequently, the enantioselective synthesis of L-carnitine has received considerable attention and several different strategies have been used. These include syntheses from chiral precursors[6] such as L-ascorbic acid, L-arabinose, *R*-malic acid, and D-galactono-1,4-lactone. Alternatively, the chiral center in L-carnitine was stereoselectively introduced by means of enzymatic asymmetric transformations[7] or via non-enzymatic chiral hydrogenations.[8] However, as each of these methods have some drawbacks, to our knowledge, L-carnitine is still prepared industrially via conventional chemical resolution of its racemate.[9] Our continued interest in developing improved procedures for the preparation of L-carnitine led us to the discovery of a microorganism that preferentially metabolized the D-enantiomer of carnitine. Herein we report the experimental procedures used for the development of a more stereoselective mutant for the preparation of L-carnitine and the biochemical basis to account for this unique stereochemical behavior.

Results and Discussion

Using conventional enrichment culture techniques, we isolated a bacterium from a sewage sample obtained from a skimming tank using mineral salts medium containing 1 % DL-carnitine HCl as a sole carbon source. This wild type culture (df-2) was identified as *Acinetobacter calcoaceticus* and was deposited with the American Type Culture Collection as ATCC 39648. The pure isolates were then tested for their abilities to utilize D- and L-carnitine as a sole carbon source on a modified Johnson's agar medium. It was observed that the isolate 'df-2' grew considerably faster on agar plates containing 0.5 % D-carnitine than on plates with 0.5 % L-carnitine. However, after four days, colonies are clearly visible on plates with L-carnitine (Figure 1).

Although the wild type strain preferentially utilized D-carnitine, it was only partially stereoselective. Hence, we subjected it to mutagenesis with a view to preparing mutants with improved stereoselectivities. This was achieved by growing *A. calcoaceticus* ATCC 39648 on a suitable medium containing DL-carnitine and then subjecting it to nitrosoguanidine (NTG) mutagenesis. The mutant is then selected on agar plates containing either D- or L-carnitine as the sole carbon source. The mutant, ATCC 39647, grew poorly on a medium containing L-carnitine as the sole carbon source even after seven days, but grew rapidly on plates containing D-carnitine (Figure 2). The selection was achieved by the replica plating method.[10]

To quantitatively assess the degree of stereoselectivity of this mutant, a series of experiments was conducted in modified Johnson's medium containing DL-carnitine. A typical growth and metabolic profile is shown in Figure 3. After 27 h, the residual L-carnitine was isolated in 93 % yield and the optical purity, expressed as enantiomeric excess (*e.e.*), was shown to be 92 %. This experiment demonstrates the effectiveness of this mutant in catalyzing this kinetic resolution by stereoselectively oxidizing the D-enantiomer of the racemate resulting in an accumulation of L-carnitine in the medium. This process is practical and amenable to scale-up.

†This paper is dedicated to Professor J. B. Jones on the occasion of his 60th birthday.

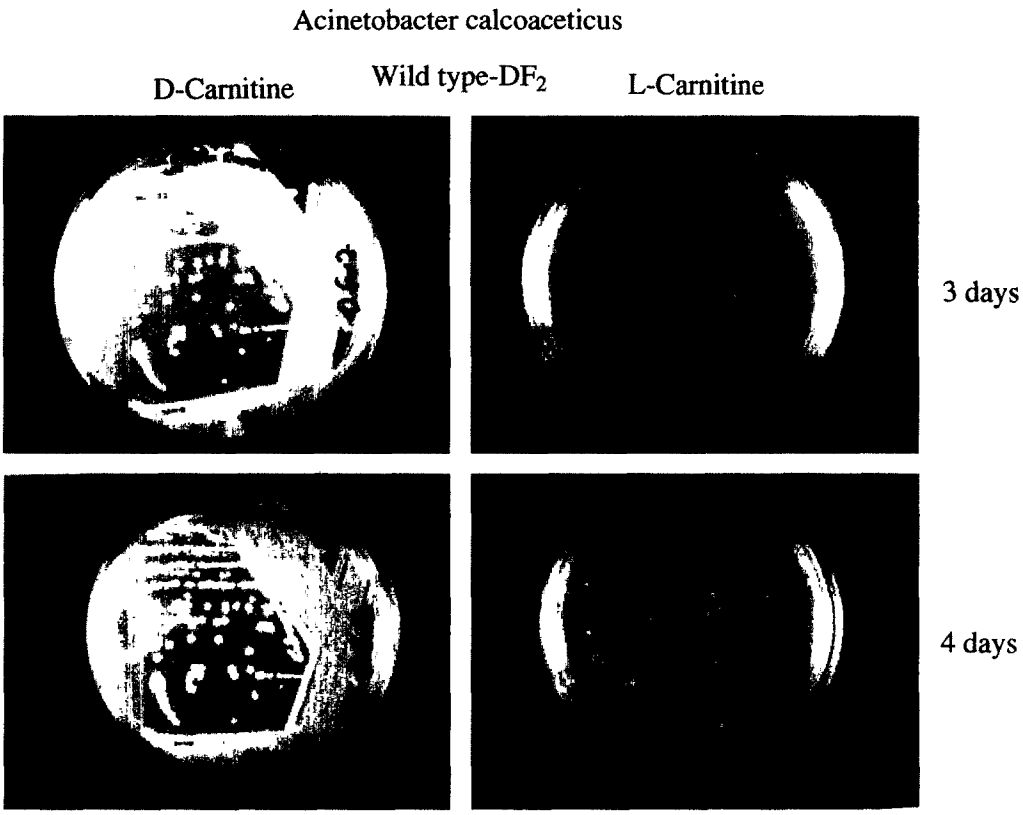


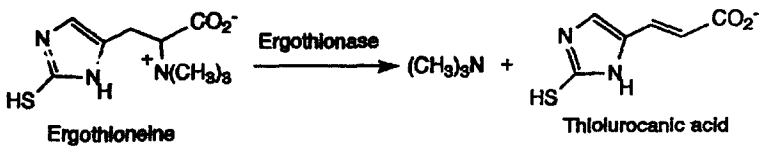
Figure 1. Growth of *A. calcoaceticus* ATCC 39648 (wild type) on agar plates containing either D- or L-carnitine after three and four days.

Acinetobacter calcoaceticus df-2 and its mutant are the first microorganisms reported with a stereochemical preference for D-carnitine. In the literature, a number of bacteria including *Pseudomonas putida* and *A. calcoaceticus* are known to selectively utilize the L-antipode when grown on a racemic mixture.[11,12] It is interesting that different strains of *A. calcoaceticus* are capable of metabolizing DL-carnitine with opposite sense of chirality. Presumably, separate enzyme/transport systems are present in the cells for the catabolism/permeation of D- and L-carnitine, and the level of expression of individual genes differs in the cells from different sources.

Until now, the pathway for carnitine degradation in microorganisms remained unclear. Cells of *P. putida* grown on carnitine were reported to produce glycinebetaine,[13] whereas formation of trimethylamine was noted for *A. calcoaceticus*.[14] In our study, when L- or DL-[methyl-^{14}C]-carnitine [$(^{14}CH_3)_3NCH_2CH(OH)CH_2CO_2H$] (Amersham) was exposed to the resting cells of *A. calcoaceticus* df-2 (wild type), stoichiometric amounts of radiolabeled trimethylamine were formed, but the rate of formation appeared to be considerably lower from L-carnitine than its racemate. Attempts to identify the degradative product(s) besides trimethylamine from the whole cell incubations were unsuccessful, presumably due to the rapid metabolism of the intermediates to CO_2 and H_2O.

Although many bacteria are known to split the C–N bond of biological intermediates to form trimethylamine, the intimate details regarding the enzymology and the mode of cleavage are fragmentary.[15] The only well-characterized reaction is that catalyzed by bacterial ergothionase[16] which may be classified as a carbon–nitrogen lyase. Studies on the catabolism of carnitine by several investigators have shown it to involve three possible mechanisms: reductive cleavage, oxidative cleavage and elimination, with the formation of β-hydroxybutyric acid,[17] or malic acid,[18] or crotonic acid, respectively. Apparently, the nature of the C_4 intermediate was the focus of these investigations.

To elucidate the degradative pathway, we used cell-free preparations of *A. calcoaceticus* ATCC 39648. The cell extract was prepared by subjecting the cell homogenate to 10,000 *g* centrifugation and $(NH_4)_2SO_4$ precipitation (35–65 %). The carnitine-metabolizing activity was measured by monitoring the amount of remaining radioactive



Scheme I.

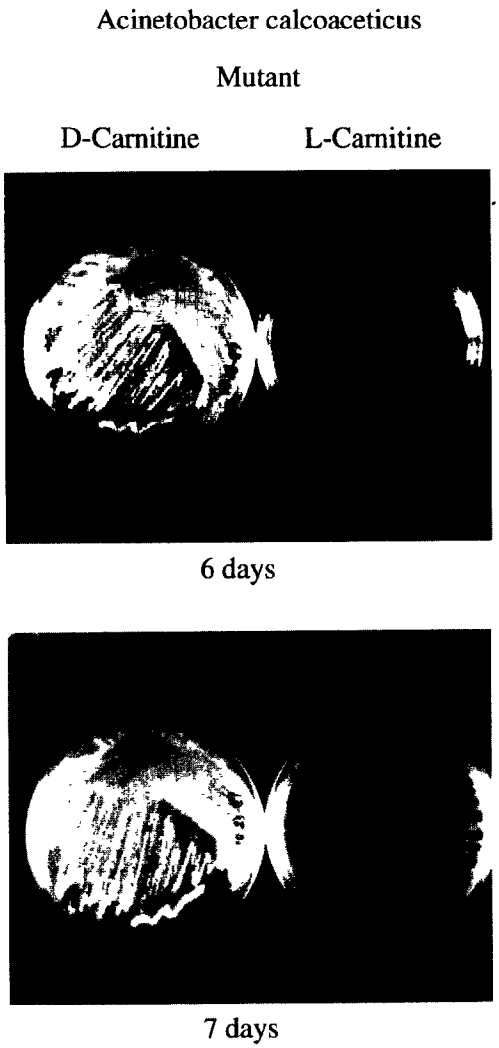


Figure 2. Growth of the *A. calcoaceticus* ATCC 39647 (mutant) on agar plates containing either D- or L-carnitine after six and seven days.

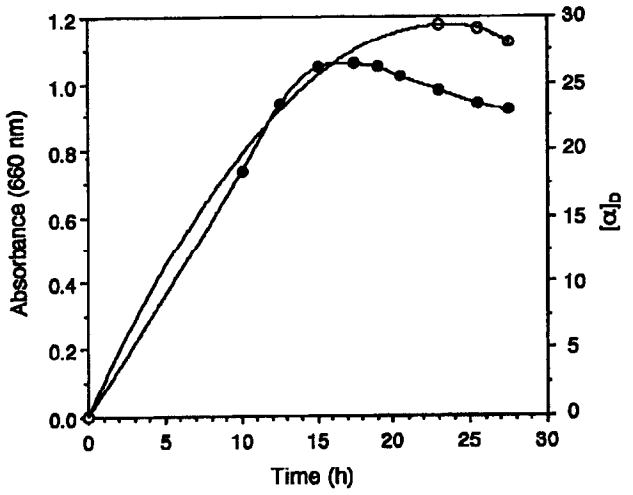


Figure 3. The stereoselective utilization of DL-carnitine (10 g/L) and growth profile of the mutant ATCC 39647. Closed circles and open circles represent the readings of OD 660 nm (diluted 1:10) and $[\alpha]_D$, respectively, of the incubation broth at indicated time intervals.

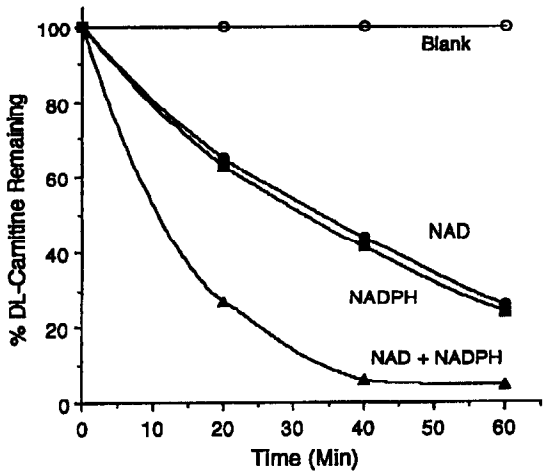
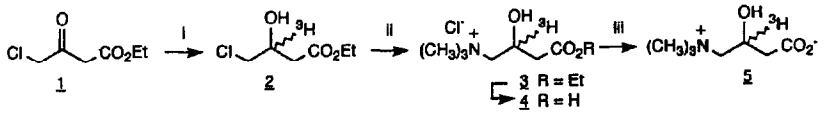


Figure 4. Coenzyme requirement of the dialyzed cell extract (wild type). The dialyzed extract (1 mL) was incubated with DL-[methyl-^{14}C]-carnitine (1 μmol; 0.1 μCi), in the presence of NAD^+ (2 μmol; closed circle), NADPH (2 μmol; closed square), or a mixture of NAD^+ and NADPH (1 mmol each; closed triangle). At the indicated time intervals, the amounts of the remaining DL-[methyl-^{14}C]-carnitine were measured.

carnitine. While the dialyzed extract was inactive by itself, upon the addition of either NAD^+ or NADPH to the incubation mixture, DL-carnitine was readily metabolized. An additive effect was noted when both coenzymes were included in the reaction mixture (Figure 4). The activity was inhibited by EDTA (1 mM), but could be restored by the addition of metal ions such as Fe^{2+}, Fe^{3+}, Co^{2+} or Mn^{2+}. Moreover, the enzyme-catalyzed formation of trimethylamine required molecular oxygen. This supposition is corroborated by the observation that under anaerobic conditions, no reaction was observed (Figure 5). Also, the metabolism of carnitine does not proceed via carnitine-CoA as CoA and ATP were not required for activity.

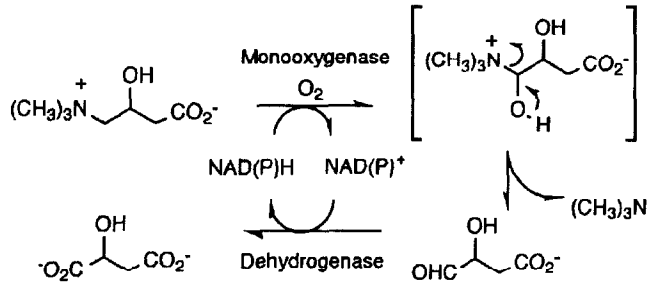
In order to identify the carbon fragment, DL-[3-^{3}H]-carnitine was synthesized according to the following series of reactions.

When the C-3 tritiated DL-carnitine was exposed to the dialyzed cell extract in the presence of NADPH, O_2 and DL-malic acid (trapping agent), all the radiolabel was found to reside in the malic acid fraction which was isolated as its dimethyl ester; no other radioactive peaks were observed.



Scheme II. i. NaB^3H_4; ii. a) $(CH_3)_3N$, b) 6N HCl; iii. Dowex-1-OH^-

This result rules out the involvement of a 3-oxo intermediate, and supports the following oxidative cleavage pathway.

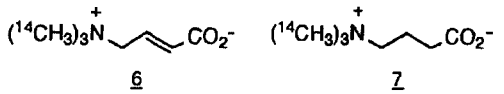


Scheme III.

The carnitine molecule is hydroxylated at C-4 by a monooxygenase in the presence of NAD(P)H and O_2, and the resulting unstable intermediate is spontaneously converted into trimethylamine and malic semialdehyde via reverse aldolization.

Oxidation of the semialdehyde regenerates the NAD(P)H with the formation of malic acid. Hence, only catalytic quantities of NAD(P)H are needed for converting carnitine into trimethylamine and malic acid. That NAD^+ was able to enhance the reaction rate may be explained by the presence of a trace amount of malic semialdehyde in the enzyme preparation. It is conceivable that NAD^+ is the preferred coenzyme for the dehydrogenase but in the absence of NADPH, malic semialdehyde levels are limiting due to reduced levels of hydroxylation. When NADPH is added, hydroxylation is more rapid and oxidation of malic semialdehyde becomes rate limiting. The turnover is more rapid when NAD^+ and NADPH are both present as compared to either coenzyme alone.

With regard to the substrate specificity, the enzyme preparation was able to utilize both [methyl-^{14}C]-4-(*N,N,N*-trimethylamino)crotonic acid (**6**) and [methyl-^{14}C]-4-(*N,N,N*-trimethylamino)butyric acid (**7**) as substrates to produce ^{14}C-trimethylamine.



Scheme IV.

Partial purification of the enzyme system indicated that it is composed of at least two dissociable protein components each of which was inactive by itself. However, the activity, as indicated by trimethylamine formation, could be restored by combining these protein fractions. It is worth noting that throughout the purification procedure, the reconstituted enzyme reacted with D- and L-carnitine at constant rate ratios (*ca* 1.5). This suggests that the metabolism of D- and L-carnitine may be mediated by the same enzyme system. A plausible explanation for the stereoselective metabolism could be that there are two separate transport systems for D- and L-carnitine. The wild type strain was able to exhibit antipodal discrimination due to the differential permeability of cell membrane to individual enantiomers. The improved selectivity through mutation may be the result of a more severe impairment of the L-carnitine transport system, while the permeation of the D-isomer is less affected. However, more in-depth studies are needed to clarify the transport mechanisms for D- and L-carnitine.

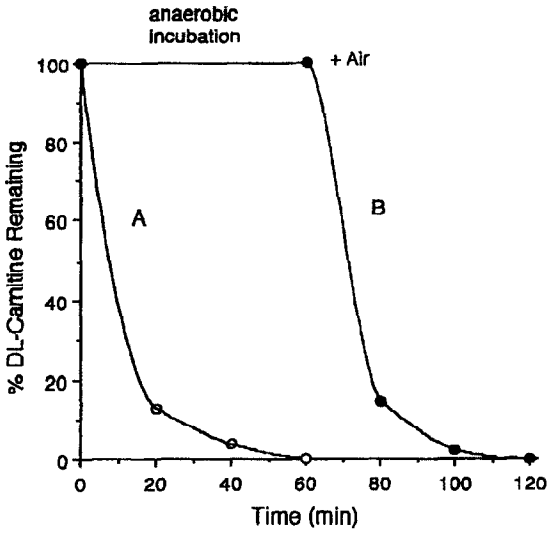


Figure 5. Requirement of molecular oxygen in the metabolism of DL-carnitine by cell extract of ATCC 39648. The dialyzed cell extract (2 mL) was incubated with DL-[methyl-^{14}C]-carnitine (2 μmol; 0.25 μCi) and NADPH (2 μmol) with and without the presence of O_2 (open and closed circles, respectively). Incubation was carried out in Thunberg tubes and anaerobiosis was achieved by five cycles of alternative evacuation and equilibration with Ar gas. Any residual dissolved oxygen was removed by glucose oxidase (100 units) in the presence of 10 mM of glucose. Both tubes were incubated at 25 °C with shaking. After 1 h, the anaerobic incubation mixture was exposed to air. At various time intervals, 200 μl aliquots of the reaction mixture were removed and added to scintillation vials containing 30 μl of 7 N NaOH. The aqueous solution was evaporated to dryness by a stream of air and then redissolved in 400 μl of distilled water. To each vial, 10 mL of scintillation cocktail was added and the radioactivity was measured.

Experimental Section

Materials

DL-[Methyl-^{14}C]-carnitine and L-[methyl-^{14}C]-carnitine were purchased from Amersham. [Methyl-^{14}C]-4-(*N,N,N*-trimethylamino)crotonic acid (**6**) was prepared by refluxing DL-[methyl-^{14}C]-carnitine in glacial acetic acid containing 50 % acetic anhydride. [Methyl-^{14}C]-4-(*N,N,N*-trimethylamino)butyric acid (**7**) was prepared by the methylation of 4-aminobutyric acid. Non-radioactive DL-, D- and L-carnitine chloride, Dowex-1-OH$^-$ resin, NAD^+, NADPH, and malic acid were products of Sigma. All solvents were glass distilled prior to use. Radioactivity was determined on a Packard scintillation counter (Model 524) which had a counting efficiency of 72 % for ^{14}C.

Isolation of the wild type (df-2) of Acinetobacter calcoaceticus *ATCC 39648*

Acinetobacter calcoaceticus ATCC 39648 was isolated from a sewage sample obtained from a skimming tank

using a selection medium via the following procedure: greasy surface water (10 mL) from a sewerage skim tank (Nine Springs treatment plant, Madison, WI) was collected and 1 ml of this sample was added to a 250 mL Erlenmeyer flask containing 50 mL of modified Johnson's medium (Medium A) [per L: Yeast extract (Difco), 50 mg; KH_2PO_4, 5.5 g; Na_2HPO_4, 10.0 g; $(NH_4)_2HPO_4$, 2 0 g; $NH_4H_2PO_4$, 1.5 g; $CaCl_2$, 15 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; $Fe_2(SO_4)_3$, 0.6 mg; $ZnSO_4 \cdot 7H_2O$, 0.2 mg; $CuSO_4 \cdot 5H_2O$, 0.2 mg; $MnSO_4 \cdot H_2O$, 0.2 mg] with 0.5 % (w/v) DL-carnitine HCl as sole carbon source, pH 6.8. The inoculated flask was incubated on a rotary shaker (250 rpm, 2" stroke, 27 °C). After 16 h, 1 mL of the turbid broth was transferred to another flask containing the same medium and reincubated on a rotary shaker for 16 h. The procedure was repeated for a third time after which the turbid broth was diluted serially using M/15 phosphate buffer, pH 7.4. An aliquot (0.1 mL) of each dilution was spread evenly over the agar surface of a Petri plate containing 1 % of DL-carnitine chloride and 2 % agar in modified Johnson's medium A. Different colony types were selected from plates that contained between 30 and 300 colonies per plate. The colonies were streaked on 1 % DL-carnitine chloride modified Johnson's medium agar plates and checked for purity. The pure isolates were then tested for their ability to utilize D-carnitine and L-carnitine as a sole carbon source on modified Johnson's medium containing 0.5 % of either D- or L-carnitine. It was observed that the isolate "df-2" grew considerably faster on D-carnitine than on L-carnitine. Hence this wild strain was chosen for the subsequent mutagenesis study.

Preparation of mutant A. calcoaceticus *ATCC* 39647 *from wild type 'df-2'*

Nitrosoguanidine mutagenesis. The wild type 'df-2', identified as *Acinetobacter calcoaceticus* (University Hospital Clinical Microbiology Laboratory, Madison, WI), was maintained on 1 % DL-carnitine chloride modified Johnson's agar medium slants and stored at 4 °C. A 250 mL Erlenmeyer flask containing 50 mL of 1 % DL-carnitine chloride modified Johnson's medium was inoculated from a slant and then incubated for 16 h on a rotary shaker (250 rpm, 2" stroke, 27 °C). To this overnight growth was added 1-ethyl-3-nitro-1-nitrosoguanidine (NTG) (Aldrich) to a final concentration of 300 μg/mL of medium. The flask was placed back on the rotary shaker and incubated for exactly 30 min. It has been previously established that 300 μg NTG/mL medium for 30 min killed approximately 99.9 % of viable cells. After 30 min exposure to NTG, the cells were collected by centrifugation (10 000 rpm, 15 min). The cell pellet was resuspended in 5 ml of modified Johnson's medium containing 1 % DL-carnitine chloride. This suspension (2 mL) was used to inoculate a 250 mL Erlenmeyer flask containing 50 mL of 1 % DL-carnitine chloride modified Johnson's medium. After the flask was incubated on a rotary shaker (200 rpm, 27 °C) for 16 h, the turbidity was determined on a Gilford UV-visible spectrophotometer (Model 240) by monitoring the absorbance at 600 nm. This data allowed the calculation of a suitable dilution to be used for plating and mutant selection.

Selection and isolation of mutant A. calcoaceticus *ATCC* 39647. Mutagenized cells, as described above, were serially diluted in 0.05 M phosphate buffer pH 7.4 and spread on to plates containing 1 % DL-carnitine chloride modified Johnson's agar medium. After incubation at 28°C for four days, the resulting colonies were replicated on modified Johnson's agar medium A containing 0.5 % L-carnitine or 0.5 % D-carnitine.

Colonies which grew well on the D-carnitine plate but poorly on the L-carnitine plate were purified by streaking onto 0.5 % D-carnitine modified Johnson's agar plates (medium A). After growth at 28 °C, individual colonies were picked from the agar plates onto slants and then evaluated in shake flasks.

Shake flasks studies

Wild type ATCC 39648. The surface growth from a one week old slant of *A. calcoaceticus* ATCC 39648 was suspended in 5 ml of saline (0.85 %) solution. This suspension (2 mL) was used to inoculate 50 mL of the modified Johnson's medium A containing 1 % DL-carnitine chloride above held in a 250 ml Erlenmeyer flask (F-1 stage). The flask was incubated at 25 °C on a rotary shaker (250 rpm/ min—2" radius) for 24 h, after which a 10 % by volume transfer was made to a 2-L Erlenmeyer flask (F-2 stage), containing 500 mL of the same medium. After 12 h on the rotary shaker, 100 mL of the medium was removed, centrifuged and then evaporated to dryness *in vacuo*. The residue was extracted with hot ethanol and the ethanolic extract was concentrated to dryness *in vacuo*. The residue was dissolved in 10 mL of 6 N HCl and the aqueous solution was concentrated to dryness. Crystallization of the residue from absolute ethanol–acetone afforded a sample of carnitine chloride (216 mg, 43 % yield) ($[\alpha]_D^{25}$ -12.0 °) (*c*, 7.2 H_2O) indicating an *e.e.* value of 0.51.

Mutant ATCC 39647. The surface growth from a one week old slant of *A. calcoaceticus* ATCC 39647 was suspended in 5 mL of medium A. This suspension (4 mL) was used to inoculate 50 mL of the modified Johnson's medium A containing 1 % DL-carnitine chloride above held in a 250 mL Erlenmeyer flask (F-1 stage). The flask was incubated at 25 °C on a rotary shaker (250 rpm/min—2" radius) for 24 h, after which a 10 % by volume transfer was made to a 2-L Erlenmeyer flask (F-2 stage) containing 500 mL of the same medium.

DL-carnitine HCl (Sigma) (20 g/L) was added and the pH adjusted to 6.8 with 4 N NaOH prior to sterilization at 121°C for 20 min.

After incubation for 44 h on the rotary shaker at 25 °C, 50 mL of the content was centrifuged to remove the cells and the supernatant was then evaporated to dryness. L-Carnitine chloride (196 mg, 38 % yield) was isolated following the aforementioned procedures. The optical purity was estimated to be greater than 96.5 % *e.e.* from the $[\alpha]_D^5$ -22.9 ° (*c*, 2.65 H_2O) value.

Studies in fermentors

The surface growth from a four day old slant of *A. calcoaceticus* ATCC 29647 was suspended in 5 mL of the following medium containing 2 % DL-carnitine chloride.

This suspension (4 mL) was used to inoculate 50 mL of the medium containing 2 % DL-carnitine chloride in a 250-mL Erlenmeyer flask (sterile). The flask was incubated at 25 °C on a rotary shaker (250 rpm/min—2" radius) for 24 h, after which 5 mL of this medium was transferred to a 250-mL Erlenmeyer flask containing 50 mL of the same medium containing 2 % DL-carnitine chloride. The flask was again incubated at 25 °C on the same rotary shaker for 24 h, after which 25 mL of the above was transferred to 250 mL of the medium containing 5 % DL-carnitine chloride in a 2-L Erlenmeyer flask. After another 24 h of incubation on a rotary shaker (250 rpm/min—2" radius), the entire flask contents (~255 mL) served as the inoculum for 2.5 L of medium containing 8 % DL-carnitine chloride in a New Brunswick 14-L stirred jar. The pH of the medium was adjusted to 6.8 with 7 N NaOH prior to inoculation. The fermentor was stirred at 355 rpm; air flow was at 4 L/min and the temperature was kept at 28 °C. After 24 h, the cells were removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The residue was dried with anhydrous ethanol by azeotropic distillation and then was refluxed for 45 min with an excess of anhydrous ethanol. The hot ethanolic extract was filtered and then evaporated to dryness under vacuum to yield a gummy residue. The gum was dissolved in water and passed through a Dowex 1-X-4 (OH) column. Evaporation of the water under vacuum afforded a white gummy residue. It was dried with anhydrous ethanol via azeotropic distillation to yield L-carnitine inner salt (75.64 g), $[\alpha]_D^{25}$ = -28.11° (*c*, 5.0 H_2O) (92.6 % yield).

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