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ORIGINAL PAPER

Role of carnitine in adaptation of *Chromohalobacter salexigens* DSM 3043 and its mutants to osmotic and temperature stress in defined medium

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Abstract

L-Carnitine is widespread in nature, but little information is available on its metabolism and physiological functions in moderate halophiles. In this study, we found that *Chromohalobacter salexigens* DSM 3043 could utilize carnitine not only as a nutrient, but also as an osmolyte. When grown at 37 °C under salt-stress conditions, the strain utilized carnitine as an osmoprotectant by enzymatically converting it into GB. When grown at low and high temperature, both carnitine and its metabolic intermediate GB were simultaneously accumulated intracellularly, serving as cryoprotectants and thermoprotectants. The genes (*csal_3172*, *csal_3173*, and *csal_3174*) which were predicted to participate in L-carnitine degradation to GB were deleted to construct the corresponding mutants. The effects of salinity and temperature on the growth rates and cytoplasmic solute pools of the *C. salexigens* wild-type and mutant strains were investigated. ^{13}C-NMR analysis revealed that GB was still detected in the Δ*csal_3172*Δ*csal_3173*Δ*csal_3174* mutant grown in a defined medium with added DL-carnitine, but not with L-carnitine, indicating that an unidentified D-carnitine degradation pathway exists in *C. salexigens*. Taken together, the data presented in this study expand our knowledge on carnitine metabolism and its physiological functions in *C. salexigens* exposed to single or multiple environmental abiotic stress.

Keywords *Chromohalobacter salexigens* · Carnitine · Glycine betaine · Compatible solute

Abbreviations

BKACE	β- Keto acid cleavage enzyme
Cm	Chloramphenicol
GB	Glycine betaine
GG	Glucosylglycerate
His	Histidine
Km	Kanamycin
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
NTA	Nitrilotriacetic acid
OD_{600}	Optical density at 600 nm
Rif	Rifampin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	Thin-layer chromatography
TMA	Trimethylamine

Introduction

L-Carnitine is widespread in nature and especially abundant in animal-derived foods (Fraenkel 1953; Bremer 1983; Jung et al. 1993; Jacques et al. 2018). Carnitine level varies in different soil and natural water ecosystems, depending on the ability of carnitine metabolism for the bacteria living in those environments (Meadows and Wargo 2015). In animals, plants, and some eukaryotic microorganisms, L-carnitine is synthesized de novo from methionine and peptide-bound lysine (Kaufman and Broquist 1977; Jung et al. 1993; Strijbis et al. 2010; Rippa et al. 2012). Prokaryotic microorganisms do not possess the carnitine synthetic pathway as described above, but some bacteria can generate L-carnitine through

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direct hydroxylation of γ-butyrobetaine by γ-butyrobetaine hydroxylase (EC 1.14.11.1) (Rüetschi et al. 1993).

So far, microorganisms have been reported to degrade L-carnitine through three different pathways, in which GB, TMA, or γ-butyrobetaine are the representative products, respectively (Fig. 1). The first pathway exists in the genera of *Escherichia*, *Salmonella*, and *Proteus*, which catalyze the conversion of L-carnitine to generate γ-butyrobetaine with crotonobetaine as the intermediate under anaerobic conditions in the presence of other carbon-and-nitrogen sources (Kleber et al. 1997). Since the carbon-and-nitrogen skeleton of L-carnitine is not assimilated by the cells, γ-butyrobetaine is found to be accumulated in the culture medium as the final metabolic product. Under aerobic conditions, some strains of the genera *Escherichia* and *Proteus* are also capable of utilizing this pathway to decompose L-carnitine, while other strains such as *Citrobacter freundii* and *Providencia rettgeri* do not (Elßner et al. 1999). In *E. coli*, the genes involved in L-carnitine degradation are located in two divergent *caiTABCDE* and *fixABCX* operons, and the presence of L-carnitine induces transcription of these genes (Buchet et al. 1998). However, the precise metabolic role of this pathway in these bacteria remains to be elucidated. The second pathway has been found in the members of the genera *Agrobacterium, Alcaligenes, Arthrobacter*, *Pseudomonas, Sinorhizobium*, and *Xanthomonas*, which are able to aerobically degrade L-carnitine, assimilating both carbon and nitrogen in the molecular backbone (Wargo and Hogan 2009; Meadows and Wargo 2015; Bazire et al. 2019). L-Carnitine is enzymatically decomposed to GB and acetoacetate in the presence of three enzymes: L-carnitine dehydrogenase, BKACE (Bastard et al. 2014), and betainyl-CoA thiolase, with dehydrocarnitine and betainyl-CoA as intermediates (Bazire et al. 2019). Then, GB can be accumulated as compatible solute to response to abiotic stresses or sequentially demethylated to form dimethylglycine, sarcosine, and glycine. The third pathway occurs in *Acinetobacter calcoaceticus* (Englard et al. 1983), *Serratia marcescens* (Unemoto et al. 1966), and *Pseudomonas putida* (Miura-Fraboni et al. 1982), which split the C-N bound of L-carnitine to produce TMA and a four-carbon (C4) unit. The resulting C4 unit is subsequently utilized as the carbon source, while trimethylamine cannot be further metabolized and released into the environment. Recently, a two-component Rieske-type monooxygenase (CntAB) from *Acinetobacter baumannii* ATCC 19,606 was shown to act on L-carnitine and its derivative γ-butyrobetaine and catalyze the oxygen-dependent cleavage of L-carnitine to TMA and malic semialdehyde. The latter was further oxidized by malate semialdehyde dehydrogenase to malic acid, an intermediate of the tricarboxylic acid cycle (Zhu et al. 2014).

In addition to its role as a nutrient, L-carnitine is accumulated by some bacteria as compatible solute in response to high-salinity or low-temperature stress. In some bacteria like *L. monocytogenes* (Lucchesi, et al. 1995), *Bacillus subtilis* (Kappes and Bremer 1998), and *Lactobacillus plantarum* (Kets et al. 1994), L-carnitine is taken up and accumulated in an unmodified form and confers osmotolerance to the high-salinity stressed or chill stressed cells, while some bacteria species in the genera *Pseudomonas* (Wargo and Hogan

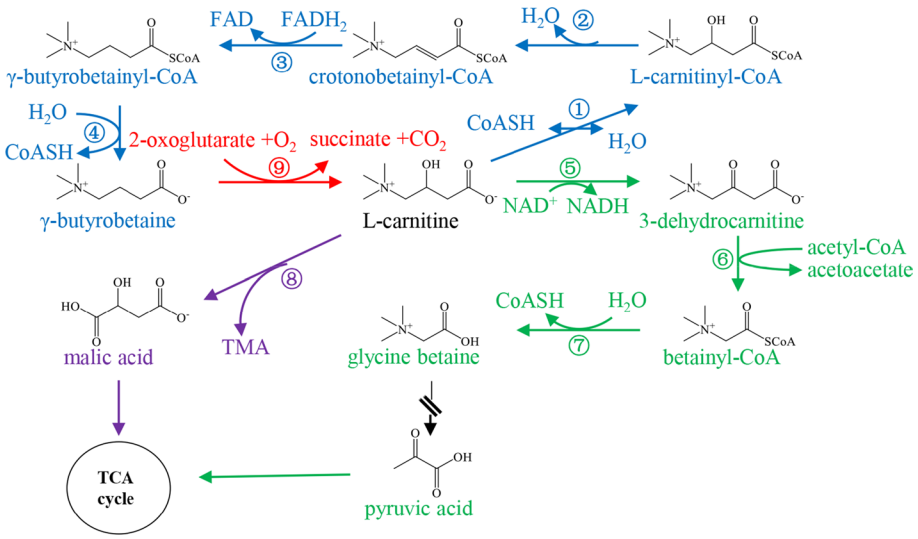


Fig. 1 Metabolism of L-carnitine in microorganisms. The enzymes involved in the metabolism include ① L-carnitinyl-CoA thiolase, ② crotonobetainyl-CoA hydratase, ③ γ-butyrobetainyl-CoA dehydrogenase, ④ γ-butyrobetainyl-CoA synthetase, ⑤ L-carnitine dehydrogenase, ⑥ dehydrocarnitine cleavage enzyme (BKACE), ⑦ betainyl-CoA thiolase, ⑧ L-carnitine monooxygenase, and ⑨ γ-butyrobetaine hydroxylase. The three different L-carnitine degradation pathways marked with blue, green, and purple colors form γ-butyrobetaine, GB and TMA, respectively, as the products, while the L-carnitine synthesis pathway from γ-butyrobetaine is marked with a red arrow

2009), *Sinorhizobium* (Bazire et al. 2019), and *Brevibacterium* (Jebbar et al. 1998) convert L-carnitine into GB after its uptake from the growth medium.

Chromohalobacter salexigens DSM 3043, a moderately halophilic bacterium formerly isolated from a solar salt facility located on Bonaire, Netherlands Antiles (Vreeland et al. 1980), can grow over a wide salinity range from 0.6 to 5.5 M NaCl (Cánovas et al. 1996), and these characteristics make it an excellent model organism for studying the mechanism of prokaryotic osmoadaptation. The intracellular accumulation of compatible solutes is of central importance for the adaptation of *C. salexigens* to osmotic stress and thermal stress (Vargas et al. 2008; Reina-Bueno et al. 2012). In the past three decades, extensive research has been conducted on the transport and metabolism of ectoine, hydroxyectoine, and GB by *C. salexigens* in response to hyperosmotic and thermal stress. In contrast, very little is known about the role and metabolism of L-carnitine in *C. salexigens*. Prior to performing the formal experiments, our preliminary results of bioinformatics analysis revealed that a gene cluster involved in L-carnitine metabolism is present in the genome of *C. salexigens* DSM 3043, implying the availability of L-carnitine in the natural habitats of this strain. In this report, we reveal for the first time the relevance of carnitine accumulation and metabolism to adaptation of *C. salexigens* to osmotic and temperature stress by investigating the effects of exogenous carnitine on cell growth and cytoplasmic solute pools of *C. salexigens* wild-type and its mutant strains under osmotic stress, temperature stress, and a combination of the two abiotic stresses.

Materials and methods

Chemicals

DL-carnitine hydrochloride, isopropyl β-$_D$-thiogalactoside, NAD^+ and Ni–NTA Sefinose resin was purchased from Sango Biotech (Shanghai, China). GB, γ-butyrobetaine, L-carnitine, L-carnitine hydrochloride, and ectoine were purchased from Merck (Shanghai, China). D-Carnitine was purchased from Dayman Chem (NeoBioscience, China). All other biochemical reagents were of analytical grade.

Bacterial strains, media, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *C. salexigens* ZW4-1, a spontaneously rifampin-resistant mutant of *C. salexigens* DSM 3043 (Shao et al. 2017), is the parent strain of all mutants used in this study and is hereafter referred to as the wild type. *C. salexigens* ZW4-1 and its derivative mutants were routinely grown aerobically at 37 °C in D6 medium, or in S-M63 mineral salt medium (per liter: NaCl, 58 g; KOH, 4.2 g; KH_2PO_4, 13.6 g; $MgSO_4$, 0.12 g; $FeSO_4{\cdot}7H_2O$, 4.0 mg; $(NH_4)_2SO_4$, 2 g; D-glucose, 3.6 g; the pH of medium was adjusted to 7.5) (Yang et al. 2020), while *E. coli* strains were normally grown in Luria–Bertani medium. When necessary, Rif, Km, and Cm were added at 50, 50, and 30 μg /ml, respectively.

Growth curves

Given growth temperatures of *C. salexigens* DSM 3043 in complex medium range from 15 to 45 °C, with optimal growth at 37 °C, and cell growth at 45 °C occurs between 0.75 and 3 M NaCl in defined media (Arahal et al. 2001; García-Estepa et al. 2006); 15 °C and 45 °C were selected as cold and heat stress temperatures in this study, while S-M63 medium containing 1, 2, and 3 M NaCl was named as low-, medium-, and high-salt medium, respectively. Cultivation of *C. salexigens* cells was carried out in 500 ml shake flask containing 75–100 mL growth medium. *C. salexigens* strains were first seeded into D6 broth containing Rif and incubated at 37 °C with shaking until late log phase. Cells were collected by centrifugation, washed twice in isotonic carbon-free S-M63 medium, and then inoculated into S-M63 medium containing different NaCl concentrations in the presence and absence of 5 mM carnitine as an exogenous additive. Then, cultures were grown aerobically at the indicated temperature (15 °C, 37 °C or 45 °C), and cell growth was monitored at 6, 12, or 24 h intervals with spectrophotometry by measuring OD_{600} values. Cultures without added carnitine served as controls, and growth curve measurements were repeated with three biological replications.

Genetic manipulation

Extraction of bacterial DNA, isolation of plasmid DNA, and purification of PCR-amplified DNA fragments were performed with the FastPure bacterial DNA isolation minikit, FastPure plasmid minikit, and FastPure gel DNA extraction minikit (Vazyme, China), respectively. Oligonucleotide primers were synthesized by TsingKe BioTech Co., Ltd. High-fidelity DNA polymerase [2 × Phanta Max Master Mix (Dye plus)] was purchased from Vazyme Biotech. Restriction endonucleases were purchased from TaKaRa Biotech (Dalian, China) and used according to the manufacturer's protocol. Colony PCR was performed as described previously (Shao et al. 2018). All of the constructed vectors and deletion mutants were verified by PCR amplification and DNA sequencing.

Extraction of intracellular osmolytes and ^{13}C-NMR spectroscopy

Strain ZW4-1 and its derivative mutants were grown to late exponential phase, and then harvested by centrifugation at

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics [a]	Source or reference
Strains		
Chromohalobacter salexigens DSM 3043	Wild type	DSMZ [b]
ZW4-1	A spontaneous rifampin-resistant mutant of *C. salexigens* DSM 3043	Shao et al. 2017
CSΔ3172	*csal_3172* deletion mutant of strain ZW4-1, Rif^rCm^r	This study
CSΔ3173	*csal_3173* deletion mutant of strain ZW4-1, Rif^rCm^r	This study
CSΔ3174	*csal_3174* deletion mutant of strain ZW4-1, Rif^rCm^r	This study
CSΔ7234	*csal_3172*, *csal_3173*, and *csal_3174 triple-gene* deletion mutant of strain ZW4-1, Rif^rCm^r	This study
E. coli strains		
E. coli DH5α	*sup*E44 Δ*lac*U169 (Φ80*lac*Z M15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	TaKaRa
E. coli S17-1 λ*pir*	*pro hsdR recA* RP4-2 (Tc::Mu)(Km::Tn7) (λ*pir*)	Shao et al. 2018
E. coli BL21(DE3)	F^- *ompT* $hsdS_B$(rB^-mB^-) *gal dcm* (DE3); T7 RNA polymerase gene under the control of the *lacUV* promoter	TianGen
Plasmids		
pK18mobsacB	Suicide vector for conjugal transfer and homologous recombination; Km^r *mob sacB* (RP4) *lacZ*	Shao et al. 2018
pACYCDuet1	Dual protein expression vector, Cm^r	Novagen
pK18mobsacB::Δ3172::cat	pK18mobsacB carrying truncated *csal_3172* gene	This study
pK18mobsacB::Δ3173::cat	pK18mobsacB carrying truncated *csal_3173* gene	This study
pK18mobsacB::Δ3174::cat	pK18mobsacB carrying truncated *csal_3174* gene	This study
pK18mobsacB::Δ3172Δ3173Δ3174::cat	pK18mobsacB carrying truncated *csal_3172* and *csal_3174* gene	This study
pBBR1MCS-2	Broad-host-range vector, Km^r	Kovach et al. 1995
pBBR1MCS-7234	pBBR1MCS-2 carrying *csal_3172*, *csal_3173*, *csal_3174* and their predicted native promoter region, Km^r	This study
pET-28a(+)	T7 promoter expression vector, Km^r	Novagen
pET28-N3173	pET28a carrying *cdhC (csal_3173)* under the control of the T7 promoter with 6×His-tag added at the N-terminus of the fusion protein (His_6-CdhC)	This study

[a] Rif^r, rifampin resistant; Cm^r chloramphenicol resistant, Km^r kanamycin resistant

[b]DSMZ, German Collection of Microorganisms and Cell Cultures

8000 × g for 20 min. After cell pellets were washed three times with isotonic NaCl solution, intracellular solutes were extracted with extraction mixture (methanol: chloroform: water = 10: 5: 4, *v/v/v*) as described previously (García-Estepa et al. 2006). For natural-abundance NMR measurements, dry extract was dissolved in 0.6 ml of D_2O, filtered through nitrocellulose membrane (pore size 0.22 µm), and then placed into 5-mm NMR tubes. ^{13}C-NMR spectra were recorded on a Bruker Advance III HD 500 MHz superconducting FT NMR spectrometer. The ^{13}C-chemical shifts of individual compounds were referenced with respect to external dimethyl sulfoxide standard ($\delta = 39.205$ ppm), and assigned by comparison with those of authentic samples or previously published chemical shift values (Cánovas et al. 1999; Yang et al. 2020).

Detection of GB and carnitine by TLC

For detection of GB and carnitine in extracts of *C. salexigens* ZW4-1, partial cellular extracts used for ^{13}C-NMR analysis were first diluted fivefold with double distilled water, and then, 2 µl of dilution was spotted onto a TLC silica gel 60 plate (precoated aluminum sheets, 5 × 10 cm, Merck, Germany). GB and L-carnitine (50 µg/µl each) purchased from Merck were dissolved in double distilled water and used as the authentic standards. After the plates were completely dried, the chromatogram was developed and stained with 0.1% bromocresol green dissolved in pure ethanol (Lechtenfeld et al. 2018). Then, the chromatoplate was photographed within 5–10 min.

Generation of *C. salexigens* mutant strains and gene complementation assays

The *Δcsal_3172*, *Δcsal_3173*, *Δcsal_3174*, and *Δcsal_3172Δcsal_3173Δcsal_3174* mutants of strain ZW4-1, designated CSΔ3172, CSΔ3173, CSΔ3174, CSΔ7234, respectively, were constructed by homologous recombination using a *sacB* counterselection strategy with pK18mobsacB as the suicide vector. Briefly, upstream and downstream flanking

regions of each target gene were PCR-amplified from the genomic DNA of *C. salexigens* DSM 3043, while a Cm resistance cassette flanked by 5′ and 3′ sequences of target gene was PCR-amplified from plasmid pACYCDuet-1, with the primer sets listed in Table 2. All PCR reactions were performed with high-fidelity DNA polymerase according to the manufacturer's recommendations. The three amplicons were cloned into the *Eco*RI-*Bam*HI site of pK18mobsacB with the ClonExpress Ultra One-Step Cloning Kit (Vazyme, China). The resulting recombinant plasmids were first transformed into chemical competent *E. coli* S17-1, and then transferred into *C. salexigens* ZW4-1 via two-parental mating. Allelic exchange mutants were screened according to the method described previously (Shao et al. 2017), and the mutants were confirmed by PCR, followed by DNA sequencing.

To complement the *Δcsal_3172Δcsal_3173Δcsal_3174* mutant (strain CSΔ7234) with functional *csal_3172*, *csal_3173*, *csal_3174* genes and their potentially native promoter, a DNA fragment containing the region from the first nucleotide downstream of the *csal_3171* stop codon to the first nucleotide downstream of the *csal_3175* stop codon was PCR-amplified from *C. salexigens* genomic DNA, and then cloned into the *Eco*RV site of pBBR1MCS-2. The obtained complementation vector was named pBBR1MCS-7234 and introduced into CSΔ7234 strain by biparental mating from *E. coli S17-1*, while pBBR1MCS-2 vector was used as a negative control and introduced into CSΔ7234 accordingly. Subsequently, strains CSΔ7234(pBBR1MCS-7234) and CSΔ7234(pBBR1MCS-2) were employed for growth experiments by separately inoculating into S-M63 medium containing L-carnitine as the sole nitrogen source to assess the ability of pBBR1MCS-7234 to restore CSΔ7234 growth.

Heterologous expression in *E. coli* and protein purification

The coding region of *csal_3173* was PCR-amplified from *C. salexigens* DSM 3043 genomic DNA using the high-fidelity DNA polymerase with primer sets N3173F and N3173R (Table 2), and cloned into the *Bam*HI site of plasmid pET28a, generating pET28-N3173, in which a six-His-tag-encoding sequence was fused into the N-terminus of *csal_3173*. After the inserted fragment in pET28-N3173 was sequenced to confirm that no point mutation was introduced during PCR amplification, the recombinant plasmid was transformed into *E. coli* BL21(DE3). A single colony of the positive transformants was inoculated into Luria–Bertani medium containing Km at 37 °C until the optical density at 600 nm reached between 0.4 and 0.6, and then, 0.05 mM isopropyl-β-D-l-thiogalactopyranoside was added to induce protein expression. After further incubation at 20 °C for 24 h with shaking, the cultures were harvested by centrifugation, washed twice with buffer A (20 mM Tris–HCl, pH 8.0), and resuspended in the same buffer. Then, cells were disrupted by sonication in an ice bath and cell debris was removed by centrifugation. The recombinant protein (His_6-Csal3173) in the supernatant was purified with Ni–NTA resin that was pre-equilibrated in buffer A. After being washed with buffer A containing 50 mM imidazole, His-tagged Csal_3173 was eluted using buffer A containing 200 mM imidazole. Pure fractions were pooled and concentrated with an Amicon Ultra 10-kDa MWCO concentrator (Merck). Protein purity was checked by SDS-PAGE and stained with Coomassie brilliant blue, and protein concentrations were determined as described previously (Bradford 1976).

Carnitine dehydrogenase assay

Carnitine dehydrogenase activity was determined by monitoring the absorbance at 340 nm due to NAD^+ reduction (NADH, $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$) (Aurich et al. 1967). The reaction mixture (1.0 ml) contained 50 mM Glycine–NaOH buffer (pH 9.0), 20 mM L- or D-carnitine, and 1.0 mM NAD^+. The reaction was initiated at 25 °C by addition of the purified His_6-Csal3173, and then, the increase at 340 nm was followed for 3 min. One unit of enzyme activity was defined as the amount enzyme catalyzing the formation of 1 µmol NADH per minute under the assay conditions.

Results

C. salexigens utilize DL-carnitine as both a nutrient and an osmoprotectant

To determine whether carnitine can function as a carbon or nitrogen source, *C. salexigens* DSM 3043 was cultivated in S-M63 mineral salt medium supplemented with 20 mM DL-carnitine, but deprived of either glucose or ammonium sulfate. Within the first 18 days of incubation, cell growth was only observed when DL-carnitine was supplemented as the sole nitrogen source, but not as the sole carbon source (Fig. 2). For the latter, the maximum value of OD_{600} was not more than 0.1. However, when the incubation time was further extended, its OD_{600} value slowly rose to 0.4 within 7 days (Fig. 2B), suggesting that carnitine can be used as the sole carbon and energy source by *C. salexigens*.

To assess a possible osmoprotective effect of carnitine on the growth of salt-stressed cells, strain ZW4-1 was grown in S-M63 medium with different salinities (1, 2, and 3 M NaCl) in the presence and absence of 5 mM DL-carnitine as an exogenous additive. When grown in medium-salinity S-M63, the growth rates of strain ZW4-1 in the presence of carnitine were higher than those without added carnitine within 8 h after inoculation. In high-salinity medium, the lag phase of ZW4-1 in the presence of carnitine was

Table 2 Primers used in this study

Primer	Sequence (from 5' to 3')[a]	Purpose
3172uF	ctatgacatgattacgaattcGATCGGCAATGGCTGGATC	Amplification of upstream flanking region of *csal_3172* for construction of pK18mobsacB::Δ3172
3172uR	**CAAAAGCACCGCCGGA**CAGTCTGGTGTCCGGCTC ATCA	
3172dF	**TGGCAGGGCGGGGCGTAA**GGCATCGACATCGAAAGC G	Amplification of downstream flanking region of *csal_3172* for construction of pK18mobsacB::Δ3172
3172dR	caggtcgactctagaggatccGGTGAGGCTACGCAATCTGC	
3172_cat_F	ATGAGCCGGACACCAGAC**TGTCCGGCGGTGCTTTTG CC**	Amplification of chloramphenicol resistance cassette from plasmid pACYCDuet1
3172_cat_R	CGCTTTCGATGTCGATGCC**TTACGCCCCGCCCTGCCA CT**	
3172-test-F	CGTGTTCGCTACCAATGCTC	Primers for screening of *csal_3172* single-gene deletion mutants
3172-test-R	GACGACAGCACCGTCAATCC	
3173uF	ctatgacatgattacgaattcCTTACCTGTGCCATCACCGG	Amplification of upstream flanking region of *csal_3173* for construction of pK18mobsacB::Δ3173
3173uR	**CAAAAGCACCGCCGGACA**GATCCAGCCATTGCCGATC	
3173dF	**TGGCAGGGCGGGGCGTAA**TGATGAGCCGGACACCAG AC	Amplification of downstream flanking region of *csal_3173* for construction of pK18mobsacB::Δ3173
3173dR	caggtcgactctagaggatccGAACATTTCGCGACGCTCAC	
3173-cat-F	ATCGGCAATGGCTGGATC**TGTCCGGCGGTGCTTTTG CC**	Amplification of chloramphenicol resistance cassette from plasmid pACYCDuet1
3173-cat-R	CTGGTGTCCGGCTCATCA**TTACGCCCCGCCCTGCCA CT**	
3173-test-F	CTTACCTGTGCCATCACCGG	Primers for screening of *csal_3173* single-gene deletion mutants
3173-test-R	GAACATTTCGCGACGCTCAC	
3174uF	ctatgacatgattacgaattcGACGTTCCCGATTCGTTGC	Amplification of upstream flanking region of *csal_3174* for construction of pK18mobsacB::Δ3174
3174uR	**CAAAAGCACCGCCGGACA**TCGATTCATGGTGGCGTCC	
3174dF	**TGGCAGGGCGGGGCGTAA**CCGTGAGCGACAAGG AGAAC	Amplification of downstream flanking region of *csal_3174* for construction of pK18mobsacB::Δ3174
3174dR	caggtcgactctagaggatccCAGCAGCTCGGTAAGGAAGG	
3174-cat-F	GACGCCACCATGAATCGA**TGTCCGGCGGTGCTTTTG CC**	Amplification of chloramphenicol resistance cassette from plasmid pACYCDuet1
3174-cat-R	TCTCCTTGTCGCTCACGG**TTACGCCCCGCCCTGCCA CT**	
3174-test-F	GACGTTCCCGATTCGTTGC	Primers for screening of *csal_3174* single-gene deletion mutants
3174-test-R	CAGCAGCTCGGTAAGGAAGG	
7234uF	ctatgacatgattacgaattcGAGCGTGACGTTCCCGATTC	Amplification of upstream flanking region of *csal_3172* for construction of pK18mobsacB::Δ3172Δ3174
7234uR	**CAAAAGCACCGCCGGACA**GTTTCGATTCATGGTGGC GTC	
7234dF	**TGGCAGGGCGGGGCGTAA**ATACCGCGTCGCTGAGCA	Amplification of downstream flanking region of *csal_3174* for construction of pK18mobsacB::Δ3172Δ3174
7234dR	caggtcgactctagaggatccCAACCTGGACACGCCAGAAG	
7234-cat-F	GCCACCATGAATCGAAAC**TGTCCGGCGGTGCTTTTG CC**	Amplification of chloramphenicol resistance cassette from plasmid pACYCDuet1
7234-cat-R	TGCTCAGCGACGCGGTAT**TTACGCCCCGCCCTGCCA CT**	
7234-test-F	GAGCGTGACGTTCCCGATTC	Primers for screening of *csal_3172*, *csal_3173*, and *csal_3174* triple-gene deletion mutant strains
7234-test-R	CAACCTGGACACGCCAGAAG	
N3173F	*gtgccgcgcggcagccatatG*CCCGCGCGGGTCGCCGTC	Amplification of *cdhC* (*csal_3173*) gene for construction of pET-N3173
N3173R	*ttgtcgacggagctcgaattc*TCAGATGCGCCCCTCGAGCC	
HB7234f	*ggtatcgataagcttgatatc*CAATGCGTCGACGCCCTC	Amplification of a 2648-bp DNA fragment containing *csal_3172*, *csal_3173* and *csal_3174* genes for construction of the complementation plasmid pBBR1MCS-7234
HB7234r	*gggctgcaggaattcgatatc*GTTCATCGCCAACGCCTTC	

[a]The sequences in lowercase letters were derived from plasmid pK18mobsacB, and the sequences in bold were derived from Cm resistance cassette in plasmid pACYCDuet-1, while the sequences derived from plasmid pET28a or pBBR1MCS-2 were indicated in italic and lowercase letters

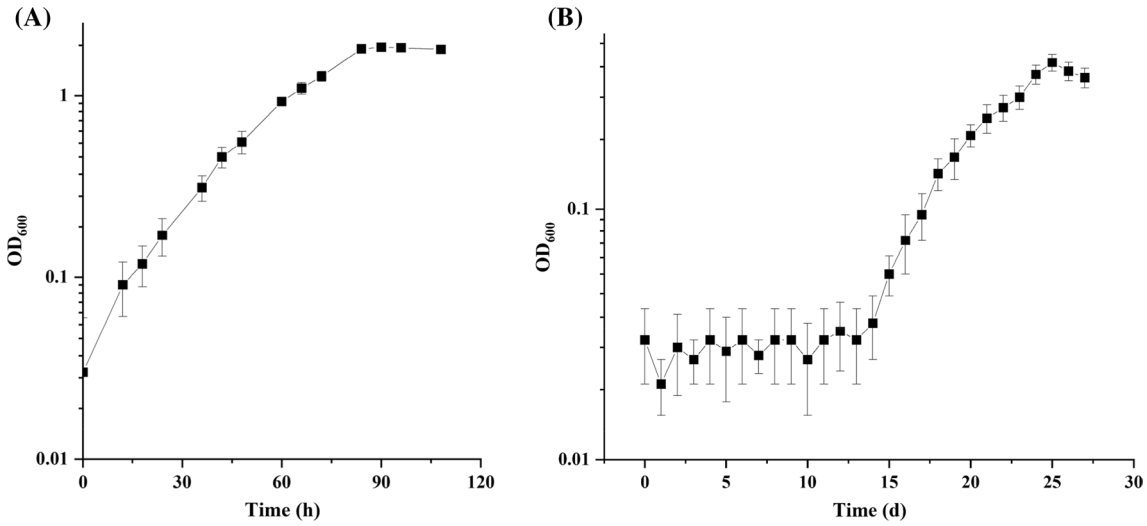


Fig. 2 Growth of *C. salexigens* ZW4-1 in a modified S-M63 medium containing 2 M NaCl, in which carnitine (20 mM) replaced ammonium sulfate **(A)** or glucose **(B)** in S-M63 medium, serving as the sole nitrogen or carbon source of this medium

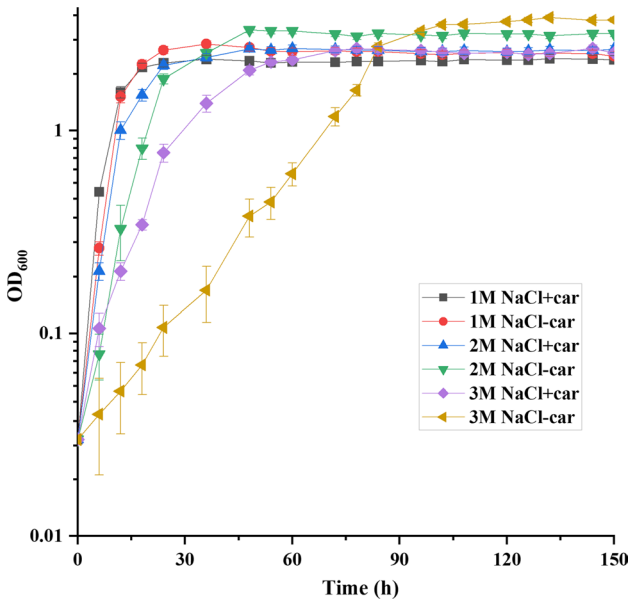


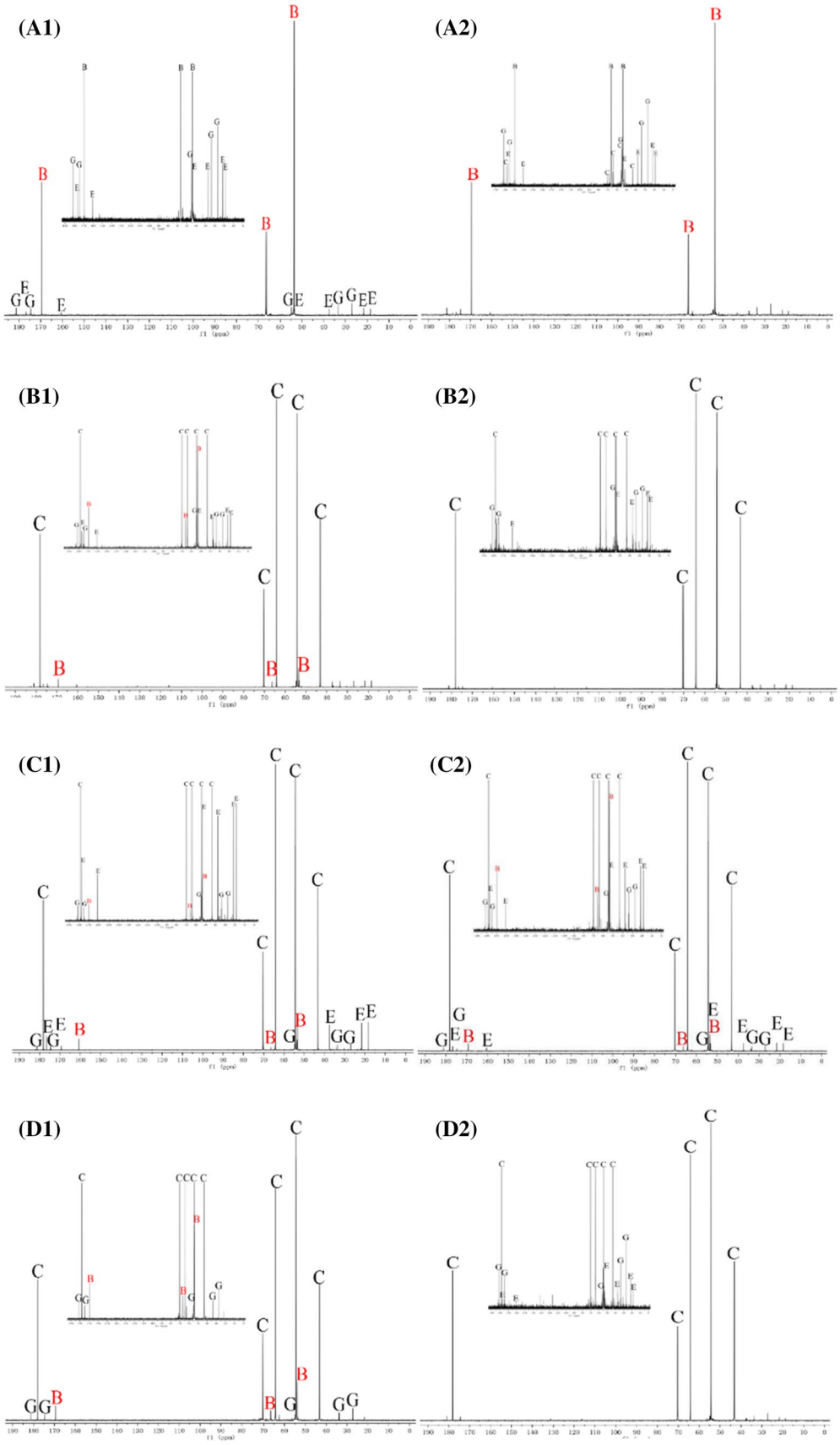
Fig. 3 Effect of DL-carnitine on the growth of osmotic-stressed *C. salexigens* ZW4-1. Cells were aerobically grown in S-M63 medium containing 1, 2, and 3 M NaCl in the presence ('+car') or absence ('- car') of 5 mM DL-carnitine, and growth was monitored over time by measuring the OD_{600}. The data shown are the means of three separately grown cultures

significantly shorten compared with those without added carnitine, and the growth rates were stimulated as well (Fig. 3). These data indicated that DL-carnitine can serve as an effective osmoprotectant for *C. salexigens*. It should be noted that the growth yields of cells with added DL-carnitine were lower than those without DL-carnitine at all tested salinities, and the molecular mechanisms for this phenomenon are currently unknown. To further determine whether the growth-promoting effects were due to intracellular accumulation of carnitine, the osmolyte pools of osmotic-stressed cells grown in medium-salinity S-M63 medium with DL-carnitine were examined by natural-abundance ^{13}C-NMR spectroscopy. Interestingly, the major NMR signals attributed to GB rather than carnitine, and minor peaks arising from ectoine and glutamate were observed as well, but no carnitine signals were detected. These data indicate that the osmoprotective effect of DL-carnitine on growth of ZW4-1 is indirect and depends on its enzymatic conversion into GB (Fig. S1).

To investigate whether the growth-promoting effects were due to the use of carnitine as carbon-and-nitrogen source by *C. salexigens*, the growth of 3043ΔbmoA strain was measured as a control. In 3043ΔbmoA strain, the GB catabolic pathway was blocked, which made it impossible for the strain to use GB as the sole carbon and nitrogen source (Shao et al. 2018). When grown in medium-salinity S-M63 with added DL-carnitine as an exogenous additive, no significant difference in the growth characteristics was observed between ZW4-1 and 3043ΔbmoA strains (data not shown), suggesting that the GB converted from carnitine was accumulated by the cells to cope with salt stress, rather than used for assimilation purpose.

Identification and sequence analysis of the genes involved in carnitine degradation to GB in *C. salexigens* DSM 3043

P. aeruginosa PAO1 and *S. meliloti* RM2011 have previously been reported to utilize L-carnitine as the sole carbon and nitrogen source and convert L-carnitine into


(A1)
(A2)
(B1)
(B2)
(C1)
(C2)
(D1)
(D2)

◂**Fig. 4** Natural-abundance ^{13}C-NMR spectra of major cytosolic solutes of *C. salexigens* CSΔ3172 **A**, CSΔ3173 **B**, CSΔ3174 **C**, and CSΔ7234 **D** grown aerobically at 37 °C in medium-salinity S-M63 supplemented with 5 mM DL-carnitine (A1, B1, C1 and D1) or 5 mM L-carnitine (A2, B2, C2, and D2). The inset is the result of amplifying the weak signals in the same spectrum. The signals were as follows: GB **B**, carnitine **C**, ectoine **E**, and glutamate **G**

GB under aerobic conditions. The genes involved in L-carnitine degradation to GB in these two strains had been identified (Wargo and Hogan 2009; Bazire et al. 2019), and functions of the associated gene products in *S. meliloti* RM2011 had been biochemically characterized (Bazire et al. 2019). Since *C. salexigens* DSM 3043 can grow aerobically on carnitine as the sole carbon and nitrogen and also has the ability to convert carnitine to GB, we hypothesized that it might possess the same carnitine catabolic route. Sequence similarity searches with BLASTp were performed using the amino acid sequences of L-carnitine dehydrogenases from *P. aeruginosa* PAO1 (NP_254073.1) and BKACE from *S. meliloti* Rm2011 (WP_010969803.1) as the query sequences to identify the gene(s) potentially involved in carnitine metabolism in *C. salexigens* DSM 3043. Two open-reading frames (ORFs), *csal_3173* and *csal_3174*, were returned in the results. Csal_3173 shares 71% amino acid sequence similarity with L-carnitine dehydrogenases from *P. aeruginosa* and exhibits homology with the N-region of the *SM2011_01638* gene product from *S. meliloti* RM2011, which has been proved to be a trifunctional enzyme with L-carnitine dehydrogenase, thioesterase, and L-carnitinyl-CoA hydrolyzing activity, while Csal_3174 has 69% amino acid sequence similarity with the BKACE from *Sinorhizobium meliloti*. The results of bioinformatics analysis with ProOpDB (http://operons.ibt.unam.mx/OperonPredictor) predicted that the two genes (*csal_3173* and *csal_3174*), as well as *csal_3172*, are oriented in the same direction and might be genetically arranged in an operon. Also, the end of *csal_3173* overlaps the beginning of *csal_3172* by 1 bp. The putative *csal_3172* product shares homology with the C-region of *SM2011_01638* product from *S. meliloti* RM2011. Thus, it is most likely that these three genes (*csal_3173*, *csal_3174*, and *csal_3172*) might serve as carnitine dehydrogenase, BKACE, and betainyl-CoA thiolase, respectively, to successively catalyze the three reactions of L-carnitine degradation to GB in *C. salexigens*. Consistent with the prediction, another ORF (*csal_3171*), adjacent to this operon, presumably encodes a putative γ-butyrobetaine dioxygenase to catalyze the conversion of γ-butyrobetaine to L-carnitine (Fig. 1), although its transcriptional orientation is predicted to be opposite to that of this operon. In fact, we found that *C. salexigens* indeed utilizes γ-butyrobetaine as the sole nitrogen source (data not shown).

Construction of gene deletion mutants and complementation assays

To determine whether each of the three genes (*Δcsal_3172*, *Δcsal_3173*, and *Δcsal_3174*) is required in the catabolism of carnitine in vivo, three single-gene deletion mutants, designated CSΔ3172, CSΔ3173, and CSΔ3174, respectively, were constructed with ZW4-1 as the parental strain. In the deletion mutants CSΔ3172, CSΔ3173, and CSΔ3174, the 336, 900, and 867-bp internal DNA fragments in the coding regions of *csal_3172*, *csal_3173*, and *csal_3174* were deleted, respectively, and a Cm resistance cassette from plasmid pACYCDuet-1 was PCR-amplified and inserted in the mutant alleles. To eliminate the polar effect caused by insertion of Cm resistance cassette, a three-gene deletion mutant (*Δcsal_3172Δcsal_3173Δcsal_3174*) were constructed and designated CSΔ7234. *C. salexigens* ZW4-1, CSΔ3172, CSΔ3173, CSΔ3174, and CSΔ7234 were separately inoculated onto S-M63 agar plates with DL-carnitine as the sole nitrogen source. Unexpectedly, CSΔ3172 was still able to grow with DL-carnitine as the nitrogen source, and growth of CSΔ3173 and CSΔ7234 was severely impaired, but growth of CSΔ3174 was completely blocked (Fig. S2). When DL-carnitine was replaced with L-carnitine, the three mutants (CSΔ3173, CSΔ3174, and CSΔ7234) lost their abilities to grow (Fig. S3).

In vitro complementation assays, strain CSΔ7234(pBBR1MCS-7234) was able to grow in S-M63 with L-carnitine as the sole nitrogen source, whereas CSΔ7234(pBBR1MCS-2) did not (Fig. S4), confirming the inability of CSΔ7234 to utilize L-carnitine as the sole nitrogen source was the result of disruption of these three genes (*csal_3172*, *csal_3173* and *csal_3174*).

Organic solute pools and growth of the four mutants with added carnitine under osmotic stress

To investigate whether the four mutants could accumulate carnitine intracellularly, the four mutants were individually inoculated into medium-salinity S-M63 medium with DL-carnitine as an exogenous additive and grown aerobically at 37 °C with shaking, cell extracts of the cultures reaching the late exponential phase were subjected to ^{13}C-NMR spectroscopy. Surprisingly, ^{13}C-NMR spectra revealed that the resonance signals of GB were present in extracts of all the mutant cells (Fig. 4). Interestingly, if L-carnitine instead of DL-carnitine was added, GB was still accumulated by CSΔcsal3172 as the dominant osmolyte (Fig. 4A), implying that the gene product of *csal_3172* might not be involved in the conversion of D- or L-carnitine to GB, or the presence of other thioesterase(s) that could replace the function of *csal_3172* in the mutant. In addition, in the presence of exogenous L-carnitine, GB

signals could still be detected in extracts of CSΔ3174, but not in extracts of CSΔ3173, although both strains accumulated carnitine as the main osmolyte (Fig. 4B, 4C). These results indicated that deletion of *csal_3173* gene completely blocked the L-carnitine-to-GB degradation pathway in *C. salexigens*, while deletion of *csal_3174* significantly reduced the conversion of L-carnitine to GB, but did not completely abolish this process. Furthermore, GB signals were also detected in extracts of CSΔ7234 cell grown in middle salinity S-M63 with DL-carnitine, but not with L-carnitine (Fig. 4D), indicating a hitherto unidentified D-carnitine metabolic pathway that is independent of these three genes, but can convert D-carnitine into GB is present in *C. salexigens*. It is worthy to note that the chemical shifts of carbon resonances of carnitine accumulated in *C. salexigens* are fully consistent with those of authentic D- or L-carnitine, but not with those of authentic DL-carnitine hydrochloride or L-carnitine hydrochloride (data not shown), suggesting that exogenous DL-carnitine hydrochloride is accumulated in *C. salexigens* as carnitine, rather than as the hydrochloride form.

To further explore the osmoprotective efficiency of DL-carnitine on the wild-type and mutant strains of *C. salexigens* grown at the optimal growth temperature, strain ZW4-1 and the four mutants were separately inoculated into the low-, medium-, or high-salinity S-M63 media with L-carnitine and cultured at 37 °C. As shown in Fig. 5, there was no significant difference in the growth rates of these strains grown in the low- and medium-salinity S-M63 media. However, when cultured in high-salinity medium, the lag phases of strains CSΔ3174 and CSΔ7234 were obviously longer than those of the other three strains; ZW4-1 grew fastest among the tested strains, and no significant difference in the growth rate and growth yield was observed between the cultures of CSΔ3172 and CSΔ3173 under these conditions.

The *csal_3173* gene product encodes an L-carnitine dehydrogenase

So far, no information is available on the gene or amino acid sequence of microbial D-carnitine dehydrogenase. The fact that *C. salexigens* DSM3043 has the ability to degrade both L- and D-carnitine to GB, and the putative *csal_3173* gene product shares sequence homology with other microbial-derived L-carnitine dehydrogenases, prompted us to investigate whether *csal_3173* encodes a carnitine dehydrogenase that can use D-carnitine as a substrate. To achieve this goal, the *csal_3173* gene was overexpressed in *E. coli* BL21(DE3) with an N-terminal His-tag and purified by Ni-chelating affinity chromatography as described above. SDS-polyacrylamide gel electrophoresis results showed that the molecular weight of the purified enzyme was approximately 40,000 (Fig. 6), which was close to the value calculated with its predicted amino acid sequence. Besides that, the purified His_6-CdhC exhibited an optimum pH of 9.0 and a specific activity of 16.75 U/mg with L-carnitine, but did not show any dehydrogenase activity toward D-carnitine.

Cold stress induces carnitine accumulation in *C. salexigens* with added DL-carnitine

It has been reported that in addition to osmotic tolerance, carnitine also confers enhanced cryotolerance in several bacterial species such as *L. monocytogenes* (Ko et al. 1994) and *Yersinia enterocolitica* (Park et al. 1995). Thus, it was interesting to determine if carnitine plays a role in cryoprotection in *C. salexigens* as well. First, growth of strain ZW4-1 cultured in low-salinity S-M63 at low temperature (15 °C) in the presence and absence of 5 mM DL-carnitine was determined. As expectedly, exogenous DL-carnitine enhanced the growth rate of ZW4-1 compared with the control without added DL-carnitine

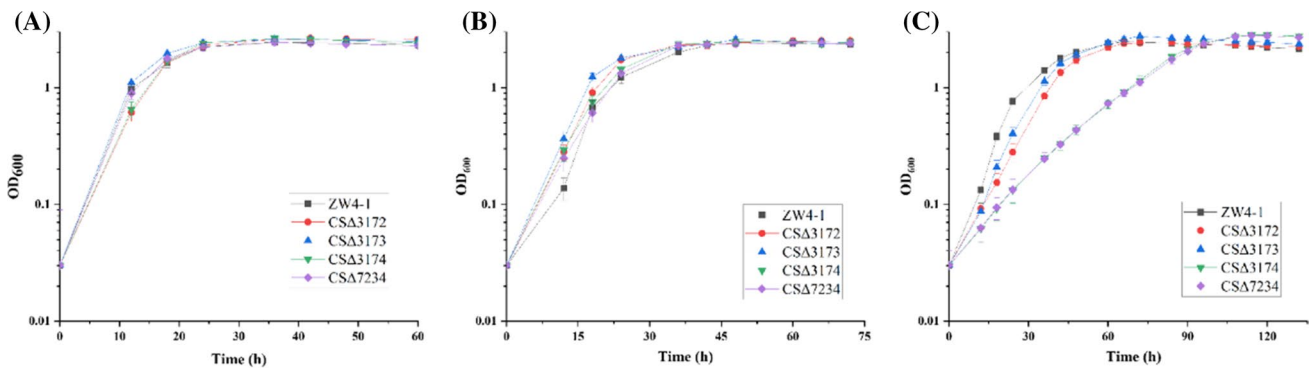


Fig. 5 Effect of DL-carnitine on the growth of the *C. salexigens* wild-type and mutant strains under osmotic stress conditions. ZW4-1 strain and the four derivative mutants (CSΔ3172, CSΔ3173, CSΔ3174, and CSΔ7234) were individually inoculated into S-M63 liquid medium containing 1, 2, and 3 M NaCl with added DL-carnitine (5 mM), and growth was monitored over time by measuring the OD_{600}. The data shown are the means of three separately grown cultures

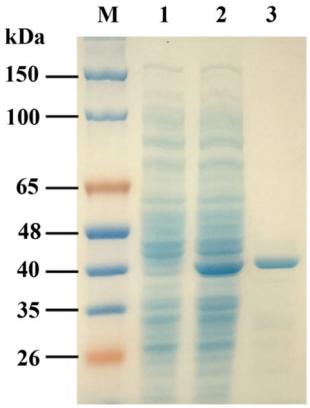


Fig. 6 SDS-polyacrylamide gel electrophoresis of His_6-Csal3173 expressed in *E. coli* BL21(DE3). lane M, molecular mass standards; lane 1, soluble fraction of *E. coli* BL21(DE3) harboring pET28a(+); lane 2, soluble fraction of *E. coli* BL21(DE3) harboring pET28-N3173; lane 3, purified His_6-Csal3173 (16 μg)

(Fig. 7A). Surprisingly, ^{13}C-NMR analysis showed that in the presence of DL-carnitine, not only GB but also carnitine was accumulated as the major osmolyte (Fig. 8A), while ectoine was accumulated as the only dominant osmolyte in the absence of exogenous osmolyte (Fig. 8B). The finding that *C. salexigens* accumulates carnitine under low-temperature conditions was unexpected, since the substrate was completely converted into GB in cells grown at 37 °C, implying that low temperature partially inhibited the conversion of carnitine to GB and triggered the intracellular accumulation of carnitine. Thin-layer chromatography (TLC) experiment further confirmed the presence of carnitine in cell extracts of ZW4-1 (Fig. 9). Then, we measured the growth curves of ZW4-1 and the four mutants grown at 15 °C in low-salinity S-M63 with added DL-carnitine, and found that the growth patterns of these strains varied differently. Among the strains tested, the growth rates of both CSΔ3173 and CSΔ3174 were generally similar and even higher than the parental strain, while the lag phase of CSΔ3172 was the longest (Fig. 7B).

To further study the osmoprotective and cryoprotective effects of carnitine on *C. salexigens* ZW4-1 and the four mutant strains (CSΔ3172, CSΔ3173, CSΔ3174, and CSΔ7234) grown at combined high salinity (3 M NaCl) and low temperature (15 °C), we measured the growth curves of these strains under these conditions, and analyzed the types of osmolytes accumulated in these cells by ^{13}C-NMR. As shown in Fig. 10A, the growth of ZW4-1 was significantly enhanced after exogenous DL-carnitine was added to growth medium. To be more specific, the culture was able to reach maximal growth within 13 days when exogenous DL-carnitine was provided, while it took 29 days for the culture to reach maximal growth in the absence of exogenous DL-carnitine. However, the final growth yield of the culture with DL-carnitine ($OD_{600} = 3.49 \pm 0.12$) was lower than that without added DL-carnitine ($OD_{600} = 3.93 \pm 0.15$). Among the tested mutants, both CSΔ3174 and CSΔ7234 exhibited a severe slow-growth phenotype, while the growth characteristics of CSΔ3172 and CSΔ3173 were similar to each other (Fig. 10B). In addition, the ^{13}C-NMR spectra revealed that carnitine, along with GB, ectoine, and other osmolytes, was detected in extracts of ZW4-1 cells grown under these conditions (Fig. S5).

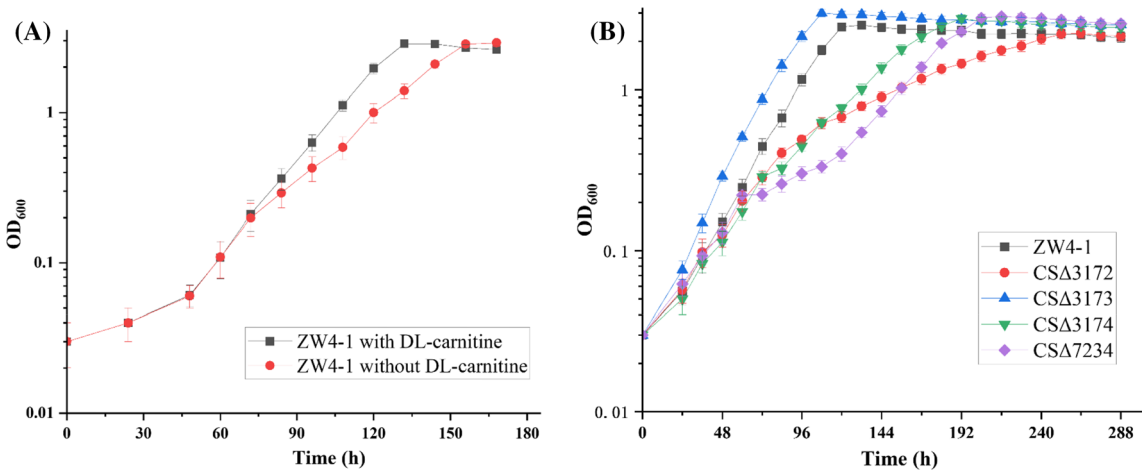


Fig. 7 Effect of exogenous DL-carnitine on the growth of *C. salexigens* ZW4-1 and its derivative mutant cells grown at 15 °C in low-salinity S-M63 with shaking at 120 rpm. **A** Growth of *C. salexigens* ZW4-1 in the presence and absence of exogenous additive DL-carnitine (5 mM). **B** Growth of *C. salexigens* ZW4-1 and its derivative mutant strains in the presence of exogenous additive DL-carnitine (5 mM). The data shown are the means of three separately grown cultures

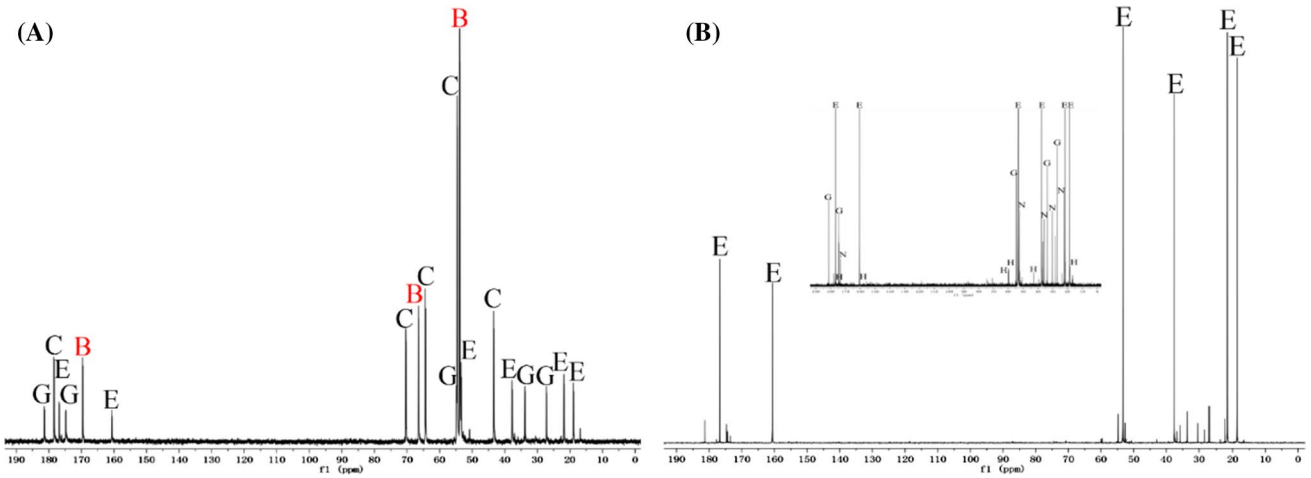


Fig. 8 Natural-abundance ^{13}C-NMR spectra of major cytosolic solutes of *C. salexigens* ZW4-1 cells grown in low-salinity S-M63 liquid medium at 15 °C with shaking at 120 rpm in the presence **A** or absence **B** of 5 mM exogenous DL-carnitine. The inset in Fig. 8B is the result of amplifying the weak signals in the same spectrum. The signals were as follows: GB **B**, carnitine **C**, ectoine **E**, glutamate **G**, hydroxyectoine **H**, and Nγ-acetyldiaminobutyric acid (N)

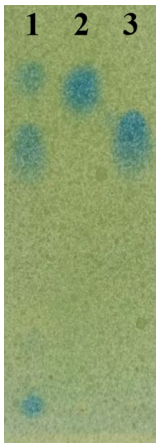


Fig. 9 TLC separation of intracellular organic solutes in *C. salexigens* ZW4-1 grown at 15 °C in low-salinity S-M63 liquid medium supplemented with 5 mM DL-carnitine as an exogenous additive. The plate was run in the system phenol:water (90:10, *v/v*) and sprayed with 0.1% bromocresol green (*w/v*) that was dissolved in pure ethanol. Lane 1: the ethanol extracts of ZW4-1 cells grown at 15 °C in low-salinity S-M63 supplemented with 5 mM DL-carnitine; lane 2: GB standard (50 μg/μl); lane 3: L-carnitine standard (50 μg/μl)

Thermal stress triggers carnitine accumulation in *C. salexigens* with added DL-carnitine

Considering that low temperatures induced carnitine accumulation and conferred chill tolerance to *C. salexigens*, we raised the question whether carnitine could also be accumulated in an unmodified form and serves as a thermoprotectant for this halophile; 45 °C was chosen as the heat stress temperature, since it is the upper limit of growth temperature for *C. salexigens* DSM 3043 (Arahal et al. 2001). As expected, the growth rate of ZW4-1 cultured at 45 °C in low-salinity S-M63 with added DL-carnitine was stimulated as compared to that without added DL-carnitine, but the growth yield was lower than that without added DL-carnitine (Fig. 11A). Among the four mutants, both CSΔ3173 and CSΔ3174 exhibited similar growth properties with ZW4-1, while CSΔ3172 and CSΔ7234 had longer lag phases and lower growth rates than ZW4-1 (Fig. 11B). The ^{13}C-NMR spectral results showed that carnitine, as well as GB was detected as the main osmolyte in cells with added DL-carnitine, but no ectoine and hydroxyectoine signals were detected (Fig. S6A), while hydroxyectoine was the main osmolyte in cells without DL-carnitine (Fig. S6B).

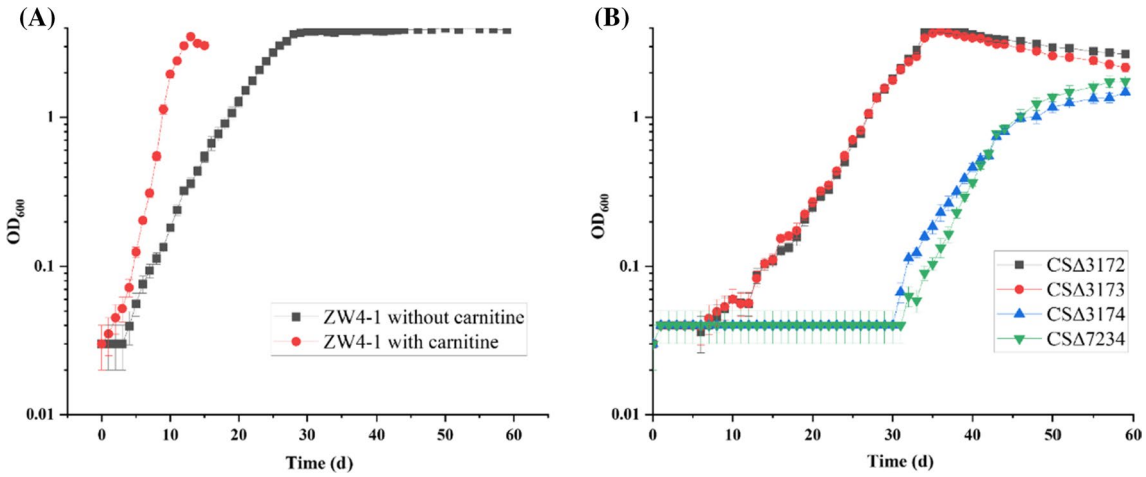


Fig. 10 Effect of exogenous DL-carnitine on the growth of *C. salexigens* wild-type and its derivative mutant strains grown at 15 °C in high-salinity S-M63 with shaking at 120 rpm. **A** Growth of *C. salexigens* ZW4-1 in the presence or absence of exogenous additive DL-carnitine (5 mM). **B** Growth of *C. salexigens* ZW4-1 strain and the four mutant strains in the presence of exogenous additive DL-carnitine (5 mM). The data shown are the means of three separately grown cultures

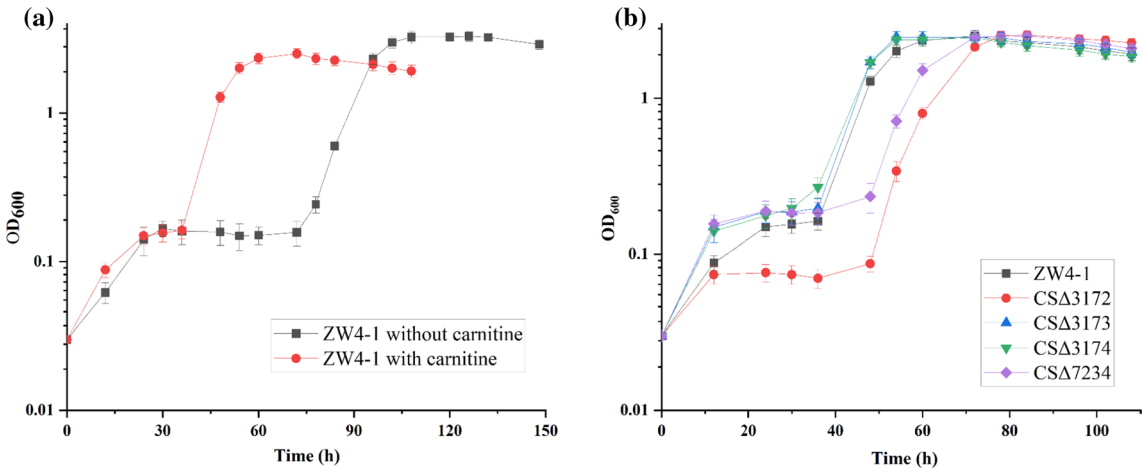


Fig. 11 Effect of exogenous DL-carnitine on the growth of *C. salexigens* ZW4-1 and its derivative mutant strains grown at 45 °C in low-salinity S-M63 with shaking at 120 rpm. **A** Growth of *C. salexigens* ZW4-1 in the presence or absence of exogenous additive DL-carnitine. **B** Growth of *C. salexigens* ZW4-1 and the four mutant strains in the presence of exogenous additive DL-carnitine (5 mM). The data shown are the means of three separately grown cultures

Similar to the case of chill stress, we further examined the stress-protective effects of carnitine on cells grown under combined high-salinity and heat-stress conditions. ZW4-1 strain did not grow when cultured at 45 °C in high-salinity S-M63 without added DL-carnitine. However, the OD_{600} value of the culture with added DL-carnitine reached the highest value of 2.5 within 72 h (Fig. 12A), indicating that exogenously provided DL-carnitine extends the upper limit of salt and heat tolerance of *C. salexigens*. In addition, ^{13}C-NMR analysis indicated that both GB and carnitine, as well as trehalose, ectoine, and Nγ-acetyldiaminobutyric acid were detected under these conditions (Fig. S7). For the four mutant strains, CSΔ3172 had a higher growth rate than CSΔ3173, but its growth yield was lower than that of CSΔ3173, while the turbidity of cultures of both CSΔ3174 and CSΔ7234 did not change within 150 h after inoculation (Fig. 12B). Because the evaporation rate of water at 45 °C was much higher than that at 37 °C, which led to a significant reduction in the volume of the medium as well as an obvious increase in salinity after incubation at 45 °C for 5 days, the growth of CSΔ3174 and CSΔ7234 cultured more than 150 h was no longer measured.

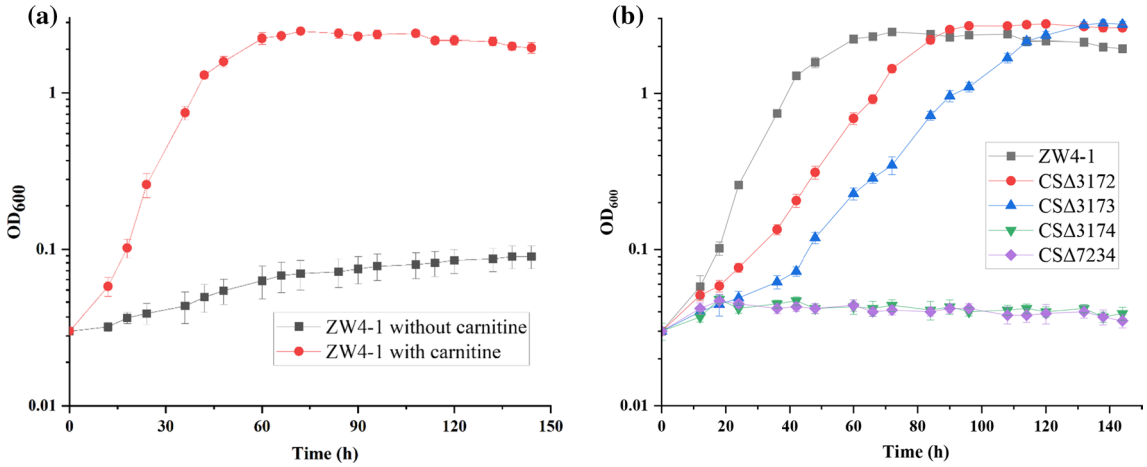


Fig. 12 Effect of exogenous DL-carnitine on the growth of *C. salexigens* ZW4-1 and its derivative mutants grown at 45 °C in high-salinity S-M63 with shaking at 120 rpm. **A** Growth of *C. salexigens* ZW4-1 in the presence or absence of exogenous additive DL-carnitine. **B** Growth of the four mutant strains in the presence of exogenous additive DL-carnitine (5 mM). The data shown are the means of three separately grown cultures

D-carnitine is converted into GB in the absence of L-carnitine in *C. salexigens*

Kleber et al. found that *Acinetobacter calcoaceticus* can use D-carnitine as a nutrient when L-carnitine is present in the incubation mixture or when the bacteria is preincubated with L- or DL-carnitine, but no growth is observed on D-carnitine as the sole carbon source (Kleber et al. 1977). To determine whether D-carnitine is accumulated intracellularly in an unmodified form or converted into GB, strain ZW4-1 was inoculated into middle salinity S-M63 supplemented with 1 mM D-carnitine as the exogenous additive. ^{13}C-NMR spectra revealed that GB, as well as ectoine was accumulated as the major osmolyte in the extracts of ZW4-1 (Fig. S8), indicating that D-carnitine can be converted into GB by *C. salexigens* even in the absence of L-carnitine.

Discussion

Genes involved in carnitine degradation to GB in bacteria

So far, the genes involved in carnitine degradation to GB were only experimentally identified in two bacterial strains, *P. aeruginosa* PAO1 (Wargo and Hogan 2009) and *S. meliloti* RM2011 (Bazire et al. 2019). In *P. aeruginosa* PAO1, a gene cluster containing *PA5385*, *PA5386,* and *PA5387* and a predicted operon containing *PA1999* and *PA2000* were found to be involved in the metabolism of carnitine to GB. The mutants with transposon insertion in each of these genes were unable to grown on carnitine as the sole carbon source. The genome of *C. salexigens* DSM 3043 lacks the homologs of *PA1999* and *PA2000* which were predicted to encode the two subunits of a putative 3-ketoacid CoA-transferase and hypothesized to be required in dacetylation of 3-dehydrocarnitine to GB and acetyl-CoA in *P. aeruginosa* (Wargo and Hogan 2009). For *S. meliloti* RM2011 strain, *SM011_c01637* which encodes a BKACE, catalyzing the formation of betainyl-CoA and acetate with 3-dehydrocarnitine and acetyl-CoA as the substrates, while *SM011_c01638* encoding a trifunctional enzyme (L-carnitine dehydrogenase/betainyl-CoA thiolase/L-carnitinyl-CoA thiolase), is actually a fused protein composed of L-carnitine dehydrogenase and thioesterase (Bazire et al. 2019). Based on the amino acid sequence homology of Csal_3172 with PA5385 from *P. aeruginosa* and SM011_c01638 from *S. meliloti*, one would have expected that a mutation in *csal_3172* could abolish the metabolism of L-carnitine in *C. salexigens*, as well. Unexpectedly, we observed that the *csal_3172* mutant retained the ability to grow on L-carnitine as the sole nitrogen source, and GB was still detected in extracts of the mutant, but the *csal_3174* mutant lose the ability to grown on L-carnitine as the sole nitrogen source, but a minor amount of GB was still detected in extracts of the mutant. In addition, the *csal_3173* mutant totally lost the ability to grown on L-carnitine as the sole nitrogen source. These data, together with the results of substrate specificity of Csal_3173, implied that the *csal_3173*-encoded carnitine dehydrogenase catalyzes the initial step of the L-carnitine catabolic pathway to produce dehydrocarnitine, then the latter is further degraded though an alternatively unknown pathway to generate GB, or other genes in the genome can replace *csal_3172* and *csal_3174* to catalyze the transformation of dehydrocarnitine to GB. It should be noted that since no homologs of *csal_3172* or *csal_3174* are found in the genome of *C. salexigens*, the

possibility that the redundant genes exist in the genome can be ruled out. Furthermore, with DL-carnitine as an exogenous additive, the mutations in *csal_3172* and *csal_3173* genes do not influence the growth of the two mutants at 37 °C in S-M63 with various salinity levels, but significantly reduce the growth rates of the two mutants grown at low and high temperature as compared to the wild-type strain, while the mutation in *csal_3174* gene significantly decreases the growth rate of the mutant under high-salinity (3 M NaCl) conditions at 37 °C, as well as at low and high temperatures, indicating that the three-gene-involved carnitine metabolic pathway is the main route for the conversion of DL-carnitine to GB in *C. salexigens*. For the triple-gene deletion mutant CSΔ7234, its growth properties at low and high temperatures were largely consistent with those of the *csal_3174* mutant (CSΔ3174), suggesting that *csal_3174* is an essential gene affecting the phenotype of CSΔ7234. Collectively, all these results suggested that the molecular mechanism of microbial metabolism of DL-carnitine to GB and the role of carnitine in adaptation of *C. salexigens* to osmotic and temperature stress are far more complex than our current understanding.

Microbial D-carnitine degradation pathway

As one of the two enantiomers of carnitine, D-carnitine is not a natural product, but a waste by-product formed in the industrial production of L-carnitine. However, various bacterial species have been found to possess the ability to degrade this compound. In contrast to L-carnitine, our knowledge about D-carnitine metabolism in microorganisms is very limited. As early as 1977, Kleber et al. found that *Acinetobacter calcoaceticus* was able to split the C–N bond of D-carnitine, if L-carnitine was present in the incubation mixture or if the bacteria were preincubated with L- or D,L-carnitine, but no growth was observed with D-carnitine as the sole carbon source (Kleber et al. 1977). Later, *Agrobacterium* sp. (DSM 8888) was found to utilize D-carnitine as the sole carbon-and-nitrogen source during aerobic growth. Two types (L-, and D-) of carnitine dehydrogenases was induced in the cultures grown on D-carnitine as the sole carbon-and-nitrogen source, but only L-carnitine dehydrogenase was generated in cultures grown on L-carnitine (Hanschmann et al. 1994). The D-carnitine dehydrogenase is a homotrimer, using D-carnitine and D, L-γ-amino-β-hydroxybutyrate as substrates, and catalyzes the reversible interconversion between D-carnitine and 3-dehydrocarnitine with NAD^+ but not $NADP^+$ as the coenzyme (Hanschmann and Kleber 1997). The degradation of D-carnitine and the occurrence of L-carnitine dehydrogenase in the same strain raises a possibility that carnitine racemase might be present in this organism. However, *Agrobacterium* sp. actually does not show any carnitine racemase activity (Hanschmann et al. 1994). On the other hand, both anaerobic growing *E. coli* 044 K74 (Jung and Kleber 1991) and aerobic growing *Pseudomonas* sp. AK1 (Mönnich et al. 1995) exhibited carnitine racemase activities, converting D-carnitine into the L-enantiomer. Unfortunately, the information about gene or protein sequences of carnitine racemases or D-carnitine dehydrogenases from microbial resources is not available. For *Pseudomonas* sp. AK1, the possibility that D-carnitine is initially catabolized by the oxidation–reduction or dehydration reaction can be ruled out, because no D-carnitine dehydrogenase or carnitine dehydratase activity was detected (Mönnich et al. 1995). For *E. coli* 044 K74, a proposal that carnitine dehydratase is responsible for carnitine racemizing activity was denied, since attempts to produce L-carnitine from D-carnitine using the purified carnitine dehydratase failed (Jung and Kleber 1991). The results present in this study show that *C. salexigens* generates GB from D-carnitine in the absence of L-carnitine and still retains the capacity even when the L-carnitine degradation pathway is completely blocked, indicating that at least an unidentified D-carnitine degradation pathway is present in this organism. In addition, the *csal_3173* gene product is proved to be an L-carnitine dehydrogenase, and cannot use D-carnitine as substrate. Thus, the D-carnitine metabolic pathways and their enzymatic background in *C. salexigens* remain unclear, and whether *C. salexigens* possesses carnitine racemase activity or D-carnitine dehydrogenase activity needs to be examined.

Accumulation of carnitine and GB in the presence of carnitine as an exogenous additive in *C. salexigens*

As one of the most effective compatible solutes, GB can be accumulated by *C. salexigens* either by transport from the medium or by transport of choline and its subsequent oxidation to GB under high-salinity conditions (Vargas et al. 2008). Here, we present the evident that another alternative pathway for GB synthesis exists in *C. salexigens*, in which the transported carnitine is partially or completely converted into GB under temperature stress and hyperosmotic stress conditions, respectively. It is known that *C. salexigens* preferentially accumulates GB when available in surrounding environments, and the accumulation of other osmolytes, either exogenously transported or endogenously synthesized, is suppressed (Calderón et al. 2004). Interestingly, our data in this study showed that the osmoprotective effects of carnitine results from its biotransformation into GB, and the presence of DL-carnitine in the growth medium results in intracellular accumulation of carnitine and its metabolic intermediate GB at low and high temperature and extends the upper limit of stress tolerance of cells grown under both high-salinity and high-temperature stress. Since *C.*

salexigens does not possess the molecular ability to synthesize carnitine de novo from methionine and lysine, the carnitine-based cold- and thermo-adaptation mechanisms for this halophile are primarily based on carnitine uptake directly from extracellular environments. Furthermore, we found that the high-salinity and thermal stressed cells contained a mixture of carnitine, GB, ectoine, and trehalose. These observations implied that when faced with two or multiple-factor stress, intracellular carnitine and its transformed GB fail to provide sufficient stress protection to the cells, which leads to the activation and upregulation of endogenous synthesis of ectoine and trehalose, resulting in accumulation of more types of compatible solutes at the same time to cope with multiple-factor stresses. For a given microorganism, the physiological response undoubtedly improves its adaptability to different ecological niches and to varying environmental factors in its habitat. Moreover, we also found that the conversion of carnitine to GB in *C. salexigens* is not inhibited by glucose in the medium. After carnitine is transported into cells, some of them are converted into GB, while the rest is accumulated directly in an unmodified form, indicating that carnitine accumulation is triggered by chill and thermal stress, and both these osmolytes (GB and carnitine) are simultaneously accumulated to confer protection against chill or thermal stress in *C. salexigens*.

Accumulation of ectoine and hydroxyectoine in the presence of carnitine as an exogenous additive in *C. salexigens*

Hydroxyectoine serves as an important osmolyte in *C. salexigens* cells grown under high-salinity and high-temperature conditions (García-Estepa et al. 2006). When grown at 37 °C (the optimal growth temperature) in defined medium in the absence of exogenous osmoprotectant, ectoine is accumulated as the main osmolyte within the entire range of salinity that it can tolerate, and its content increases with increasing concentrations of NaCl in growth medium (Cánovas et al. 1997; García-Estepa et al. 2006). The results in this study showed that in the absence of exogenous DL-carnitine, ectoine is the major osmolyte in cells grown at 15 °C, while hydroxyectoine is the predominant osmolyte in cells grown at 45 °C. These observations implied that ectoine and hydroxyectoine play an essential role in chill and thermal stress protection, and were in agreement with the previous report (García-Estepa et al. 2006). In addition, we found that accumulation of carnitine in *C. salexigens* severely suppressed hydroxyectoine accumulation under high-temperature conditions, as well as under combined high-salinity and high-temperature conditions. However, addition of D-carnitine as an exogenous additive to S-M63 medium has no significant inhibitory effect on intracellular accumulation of ectoine, and the exact molecular mechanism for this phenomenon needs to be further studied.

Accumulation of GG and trehalose in the presence of carnitine as an exogenous additive in *C. salexigens*

Both GG and trehalose belong to sugar-type compatible solutes, and are considered to be synthesized under nitrogen-limiting conditions to cope with environmental abiotic stress in many bacteria. In *C. salexigens*, GG synthesis mainly occurs at the optimum growth temperature (37 °C) with salinity range from 0.5 to 1.5 M NaCl (Calderón et al. 2004) or when ectoine synthesis or GB degradation is impaired (Cánovas et al. 1997; Yang et al. 2020), but does not happen at high salinity (2.5 M NaCl). Trehalose synthesis is triggered by a combination of high salinity (2.5 M NaCl) and high temperature (45 °C) (García-Estepa et al. 2006; Reina-Bueno et al. 2012), or when ectoine synthesis or GB degradation is blocked (Cánovas et al. 1997; Yang et al. 2020), but does not occur at the optimum growth temperature (37 °C) (Calderón et al. 2004). The presence of GB or choline in the growth medium can completely repress the accumulation of both trehalose and GG (Cánovas et al. 1996), but exogenous dimethylglycine can induce the synthesis of both osmolytes (Yang et al. 2020). In this work, GG was detected in cells grown in low-salinity M63 at 45 °C in the presence and absence of exogenous DL-carnitine, while trehalose was accumulated in cells grown at combined high salinity (3 M NaCl) and high temperature (45 ℃) with added DL-carnitine, indicating that GG synthesis is induced by high temperature at low- and medium-salinity, and intracellular accumulation of carnitine does not inhibit the synthesis of GG and trehalose, although GB is simultaneously accumulated under these conditions. Moreover, neither GG nor trehalose was synthesized by the cells grown in S-M63 at low temperature (15 ℃) in the presence and absence of exogenous DL-carnitine, suggesting that these two osmolytes serve as thermoprotectants, but not as cryoprotectants in *C. salexigens*.

Conclusions

Our results presented in this work ascribe an important physiological function about carnitine accumulation and metabolism in adaptation of *C. salexigens* to high osmotic stress and permanently cold and heat environments, and indicate that the roles of carnitine in bacterial adaptation to external stress conditions are much broader and are not limited to conferring protection against osmotic stress or chill stress.

However, the molecular mechanism through which cellular stress protection is achieved by carnitine in *C. salexigens* remains to be explored in the future studies.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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