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ORIGINAL PAPER

Establishment of a markerless gene deletion system in *Chromohalobacter salexigens* DSM 3043

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Abstract *Chromohalobacter salexigens* DSM 3043 can grow over a wide range of salinity, which makes it as an excellent model organism for understanding the mechanism of prokaryotic osmoregulation. Functional analysis of *C. salexigens* genes is an essential way to reveal their roles in cellular osmoregulation. However, the lack of an effective markerless gene deletion system has prevented construction of multiple gene deletion mutants for the members in the genus. Here, we report the development of a markerless gene deletion system in *C. salexigens* using allelic exchange method. In this system, the in vitro mutant allele of target gene was inserted into a pK18mobsacB-based integrative vector pMDC21, which contained a chloramphenicol resistance cassette as the positive selection marker and a *sacB* gene from *Bacillus subtilis* as the counterselectable marker. To validate this system, two single-gene deletion mutants and a double-gene deletion mutant were constructed. In addition, our results showed that growth of the merodiploids and sucrose screening at 25 °C were more effective to decrease the occurrence of spontaneous sucrose resistance colonies than at higher temperature (30 or 37 °C), and growth of the merodiploids in mineral salt medium instead of the complex medium was critical to increase the recovery rate of deletion mutants.

Keywords *Chromohalobacter salexigens* · Allelic exchange · Homologous recombination · Markerless deletion · *sacB*

Introduction

Chromohalobacter salexigens DSM 3043 is a metabolically versatile, moderately halophilic bacterium that can grow over a wide range of salinity, in the presence of NaCl concentrations ranging from 0.5 to 3 M NaCl in M63 glucose-defined medium and from 0.1 to 4 M NaCl in complex medium, which makes it as excellent model organism for studying prokaryotic osmoadaptation (Arahal et al. 2001; Canovas et al. 1996; Vargas et al. 2008; Vreeland et al. 1980) In addition, *C. salexigens* strains have recently received a considerable interest as resources for ectoine, hydroxyectoine, and poly-hydroxybutyrate production (Vargas and Nieto 2004; Ventosa and Nieto 1995; Yin et al. 2015). Because most of them are very easy to grow and maintain in the laboratory and their nutritional requirements are very simple, increasing effects have been made to use halophiles as cell factories for bioprocessing with advantages of the lower energy and less fresh water consumption (Yin et al. 2015).

The complete genome sequence of *C. salexigens* DSM 3043 had been determined by the Joint Genome Institute of the US Department of Energy (Copeland et al. 2011), which presents an opportunity for understanding its characteristic metabolic network that enable it to grow in the presence of high concentrations of NaCl inhibitory to most non-halophiles. To determine gene function, efficient

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methods for generating mutants are needed. Besides of physical radiation and/or chemical reagents, two biological methods have been employed for the construction of *C. salexigens* mutants. The first is random mutagenesis with transposon Tn1732 (Canovas et al. 1997; Kunte and Galinski 1995). Tn1732 is a derivative of Tn1721, consisting of the "basic transposon" of Tn1721 that encodes the transposition functions (transposase, tnpA, and resolvase, tnpR, genes). The suicide vector pSUP102-Gm carrying Tn1732 could be delivered into *C. salexigens* via *E. coli* SM10 mediated conjugation to disrupt the target genes in the chromosome of *C. salexigens*. In our laboratory, the suicide plasmid pUTminiTn5 (Manilla-Pérez et al. 2010) carrying miniTn5 was used for insertional mutagenesis of *C. salexigens* via *E. coli* SM10 mediated conjugation. Although several salt-sensitive mutants were obtained, the sequence analysis results showed that the distribution of interrupted genes was limited, indicating the presence of "hot spots" for Tn5 integration in *C. salexigens* (unpublished results). Other transposons belonging to the Tn7 and Tn10 families had been used for insertional mutagenesis in *C. salexigens*, but it was unsuccessful (Argandoña et al. 2012). These results cause severe limitation to the use of transposons in *C. salexigens*. The second approach is targeted mutagenesis by allelic exchange between the target genes and in vitro-modified alleles which can be manipulated by introducing deletions, insertions or base replacements. In *C. salexigens*, the knockout mutants reported were usually generated by antibiotic marker allelic exchange (García-Estepa et al. 2006; Reina-Bueno et al. 2012; Rodríguez-Moya et al. 2010; Salvador et al. 2015; Tokunaga et al. 2004). No matter what kinds of strategies used as mentioned above, a cassette containing antibiotic resistance gene was ultimately retained in the modified organism, which may result in some limitations in complementation analysis or in the construction of multiple deletion mutations in a strain. Because the number of available antibiotic markers that could be used for halophiles is much lower than that for non-halophiles, multiple gene deletion in *C. salexigens* with marker exchange method is extremely difficult. Besides that, insertion of antibiotic resistance cassettes may cause polar effects on the expression of downstream genes at the target locus, especially for the polycistronic operons. In order to overcome this drawback, a more efficient method to delete target genes or genomic regions without leaving selection markers or foreign DNA sequences behind should be established.

Recently, CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 system, a novel gene editing tool, has been successfully applied to gene targeting in microorganisms, plants and animals (Bortesi et al. 2016). This system usually has two main components, Cas9-nuclease and a single chimeric guide RNA (sgRNA), and can modify multiple genes in the chromosome at one time with the short protospacer sequences of the sgRNA (Ran et al. 2013). To apply this technique for the genome editing of *C. salexigens* DSM 3043, the main technical barrier to be resolved is how to transform the sgRNA into *C. salexigens* cells. However, the methods for direct transformation of DNA or RNA fragments into *C. salexigens* cells by electroporation or other treatments are not available now, which make it impossible for construction of *C. salexigens* mutants with this system at the current situation. In this report, we described using the suicide vector pMDC21, a derivative of pK18mobsacB to generate unmarked, in-frame deletion mutants in *C. salexigens* via allelic exchange. To demonstrate the reliability of our system, two single-gene deletion mutants and one double-gene deletion mutant of *C. salexigens* DSM 3043 were constructed, and the factors to reduce the ratio of the spontaneous sucrose resistance colonies and improve the recovery rate of desired recombinants were investigated as well.

Materials and methods

Strains, plasmids, primers, and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* DH5α and S17-1 strains were routinely grown at 37 °C under aerobic condition in Luria–Bertani (LB) medium (per liter: tryptone 10 g, yeast extract 5 g, NaCl 10 g, pH 7.5) (Sambrook et al. 1989), or in M63 medium (per liter: $(NH_4)_2SO_4$ 2 g, KH_2PO_4, 13.6 g, $FeSO_4{\cdot}7H_2O$ 0.5 mg, $MgSO_4$, 0.12 g, D-glucose 3.6 g, adjust pH to 7.5 with 10 M KOH). *C. salexigens* DSM 3043 and its derivative strains were routinely grown at 37 °C with aeration in D5 medium (per liter: NaCl 50 g, $MgSO_4{\cdot}7H_2O$ 5 g, tryptone 5 g, and yeast extract 3 g, pH 7.5), or in M63 medium (pH 7.5) supplemented with 0.86 M NaCl. Solid media were obtained by adding 20 g of agar powder (DaMao, TianJin, China) per liter. LB medium containing 4 mM $MgSO_4$ and additional 0.35 M NaCl was named as LB-Mg medium, while LB medium lacking the ingredient NaCl was named as YET medium. When required, the media were supplemented with the substrates and antibiotics at the following concentrations: 20 mM glycine betaine, 20 mM dimethylglycine (DMG), 20 mM sarcosine, 20 mM glycine, 20 mM glucose, 292 mM sucrose, 70 μg ml^{-1} chloramphenicol (Cm), 50 μg ml^{-1} kanamycin (Km), and 50 μg ml^{-1} rifampicin (Rif).

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant feature(s)	Source or reference
C. salexigens strains		
DSM 3043	Wild type	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany)
ZW4-1	Spontaneous rifampicin-resistant mutant of DSM 3043	This study
3043*Δ1415*	ORF *csal_1415* in-frame deletion mutant of *C. salexigen* ZW4-1	This study
3043*ΔsarC*	*sarC* gene in-frame deletion mutant of *C. salexigens* ZW4-1	This study
3043*Δ1415Δsar*	*csal_1415* and s*arC* double-gene in-frame deletion mutant of *C. salexigens* ZW4-1	This study
Escherichia coli strains		
DH 5α	F^- Φ80d*lacZDM15* Δ(*lacZYA-argF*)*U169 recA1 hsdR17*($r_K^- m_K^+$) *supE44* Δ^- *thi-1 gyrA relA1*	(Sambrook et al. 1989)
S17-1	*thi pro hsdR hsdM*$^+$ *recA* Tp^r Sm^r, conjugation strain	(Simon et al. 1983)
Plasmids		
pK18mobsacB	Km^r *mob sacB*	(Schäfer et al. 1994)
pACYCDuet-1	Dual proteins expression vector, Cm^r	Novogen
pMDC21	Cm^r *mob sacB*	This study
pMDCΔcsal_1415	pMDC21 harboring truncated *csal_1415* gene	This study
pMDCΔsarC	pMDC21 harboring truncated *sarC* gene	This study

General DNA manipulations

Routine DNA manipulations were carried out as described previously (Sambrook et al. 1989). Genomic and plasmid DNAs were extracted from bacteria using the Ezup Column Bacteria Genomic DNA Extraction Kit (Sangon, China) and SanPrep Column Plasmid Mini-Preps Kit (Sangon, China), respectively. After electrophoresis, DNA fragments were purified using the SanPrep Column DNA Gel Extraction Kit (Sangon, China). T_4 DNA ligase and restriction enzymes were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China) and used as recommended by the manufacturer's instructions. Amino acid and DNA alignments were done using the software DNAMAN version 8.0 (Lynnon Biosoft). Polymerase chain reaction (PCR) was normally conducted in 20 μl volume using the Pfu PCR MasterMix (TianGen, China) according to the manufacturer's instructions. To perform a colony PCR, a small amount of bacterial colony were resuspended in 20 μl of double-distilled water, boiled for 5 min, and centrifuged at 6000×*g* for 5 min. The resulting supernatant was used as template in the following PCR reaction.

Plasmids construction

The primers used in this study are listed in Table 2, and all DNA sequences used are retrieved from the NCBI web page (https://www.ncbi.nlm.nih.gov/). All of the plasmid regions obtained by PCR were sequenced to verify that no mutations were introduced.

The suicide or integrative plasmid was constructed with pK18mobsacB as the backbone. The Cm resistance cassette was PCR amplified from pACYCDuet-1 using the primers Cm-F and Cm-R. The resulting 1054-bp PCR product was gel-purified, and cloned into *Bgl*II-*Nco*I

Table 2 Oligonucleotides used in this study

Primers	Sequence (5′–3′)[a]
Cm-F	TGATGGCGCAGGGGATCATGTCCGGCGGTGCTTTTGCC
Cm-R	TTCGGCAAGCAGGCATCGTTACGCCCCGCCCTGCCACT
1415-uF	CCGGAATTCTGCCATTCATCAGCGAT (*Eco*RI)
1415-uR	GAAAGAGAATCACCGTACCCGAGAGCA
1415-dF	GGGTACGGTGATTCTCTTTCCTTGGTCAGACA
1415-dR	CGCGGATCCAGCGAACAATGATTCCGC (*Bam*HI)
sar-uF	CCGGAATTCTGGACTGGCATTACACGC (*Eco*RI)
sar-uR	GGATGTATTTCTCGAGCACTGCCACATT
sar-dF	AGTGCTCGAGAAATACATCCGCAAGACGG
sar-dR	CGCGGATCCCACGGTAGTCGGTACGAAAC (*Bam*HI)
1415-test-F	CTCCTGCGAATACAACAGC
1415-test-R	TGAGGAAGTGGTTTAGGCG
sar-test-F	ATGCAACGTTATTCAGGCTT
sar-test-R	GTGACCGAGTTGAAATACACCT
sacB-F	TCGTCTTTGCATTAGCCGGA
sacB-R	CGCCTTGGTAGCCATCTTCA

[a] Restriction endonuclease sites are underlined

double-digested pK18mobsacB using the ClonExpress® II One Step Cloning Kit (Vazyme, Nanjing, China), creating pMDC21 (GenBank accession number: KY406738) (Fig. 1).

The open reading frame (ORF) Csal_1415 encodes a putative Na^+/H^+ antiporter (NhaC) in *C. salexigens* DSM 3043 (NCBI reference sequence: WP_011506715.1). pMDCΔcsal_1415 was constructed for the *csal_1415* in-frame deletion in strain ZW4-1. Two primer pairs (primers 1415-uF and 1415-uR; primers 1415-dF and 1415-dR) were used to amplify the upstream homologous region (UHR) and downstream homologous region (DHR) of ORF *csal_1415* with *C. salexigens* ZW4-1 genomic DNA as template. The two PCR amplicons, both 955-bp in length, were then fused together by splicing by overlap extension PCR (SOE-PCR) (Horton 1997). For this purpose, the 3′ end of the upstream fragment 1415-uR include a 20-bp overhang with homology to the 5′ end of the primer 1415-dF. The resulting PCR fragment, 1890-bp in length, was gel-purified, double-digested with *Eco*RI and *Bam*HI, and ligated into the *Eco*RI and *Bam*HI sites of pMDC21, yielding pMDCΔcsal_1415.

pMDCΔsarC was constructed for the *sarC* (*csal_0998-1001*) in-frame deletion in strain ZW4-1. Two pairs of primers (primers sar_uF and sar_uR; primers sar_dF and sar_dR) were used to amplify the UHR (961 bp) and DHR (958 bp) of the *sarC* gene (encoding for a putative sarcosine oxidase), respectively. The two amplicons were then fused together by SOE-PCR with primers sar_uF and sar_dR to generate a 1899-bp fragment, which was gel-purified, double-digested with *Eco*RI and *Bam*HI, and ligated to pMDC21, yielding pMDCΔsarC.

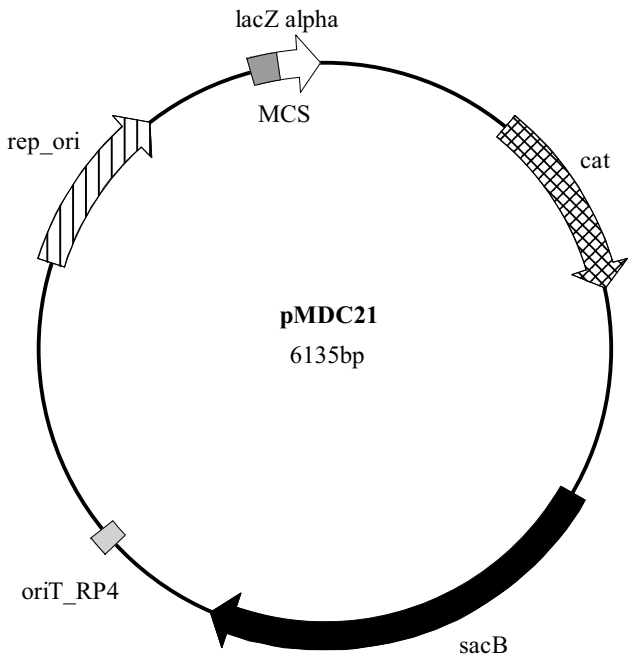

Fig. 1 Plasmid map of the integrative vector pMDC21. pMDC21 has the following features: a pMB1-based origin of replication (rep_ori, suicide plasmid for *C. salexigens*), the origin of transfer for the RP4 plasmid (oriT_RP4, for conjugal transfer between *E. coli* and *C. salexigens*), the pUC18 multiple cloning site (MCS, the mutated allele to be introduced into *C. salexigens* can be inserted into this site), *lacZ* alpha (encoding for α-peptide of galactosidase), *cat* (chloramphenicol resistance gene, positive selection marker), *sacB* (levansucrase-encoding gene from *B. subtilis*, negative selection marker). The rep_ori, *lacZα*, *cat* and *sacB* are represented by *arrows* showing the direction of replication or transcription, while MCS and oriT_RP4 (origin of transfer for the RP4 plasmid) are represented by a *bar*

Generation of single- and double-gene deletion mutants

C. salexigens ZW4-1 *csal_1415* unmarked, in-frame deletion mutant was constructed by a two-step process: allelic integration, and recombinative excision. First, the allelic exchange plasmid pMDCΔcsal_1415 was transformed into competent *E. coli* S17-1 cell by the routine $CaCl_2$ method (Sambrook et al. 1989), and subsequently transferred into *C. salexigens* ZW4-1 strain by biparental filter mating, which were performed as follows. The donor *E. coli* S17-1 strain harboring pMDCΔcsal_1415 was aerobically grown at 37 °C in LB broth supplemented with chloramphenicol until the late exponential growth phase, and the recipient ZW4-1 strain was grown in D5 medium containing rifampicin until the early stationary phase under the same culture conditions. Both *E. coli* and *C. salexigens* cells were separately harvested by centrifugation (5400×*g*, 5 min), washed two times with 10 mM $MgSO_4$, resuspended in the same solution, and mixed at a ratio of 2:1. Then, 50 μl (equivalent to approximately 10^9 cells) of the mating mixtures was placed onto a nitrocellulose filter disk (0.45 μm pore size, 25 mm diameter) that had been placed aseptically on LB-Mg agar plate. After incubation at 37 °C for 12–16 h, the cells on the filter disk were resuspended in 500 μl of 10 mM $MgSO_4$, serially diluted, and plated on D5 plates plus Rif (to eliminate the donor bacteria) and Cm (for selection of transconjugants with plasmid integration by the single homologous recombination event). After incubation for 48 h at 37 °C in the dark, the colonies growing on the plates were selected. Colony PCR was performed with the primers sacB-F and sacB-R to detect the plasmid backbone. For the second step, one of the merodiploids was inoculated in D5 medium without antibiotics for 18 h at 30 °C. To select for bacteria that had undergone a second recombinant event, the culture was serially diluted, spread onto D5 plates supplemented with sucrose, and incubated at 30 °C for 48 h. The colonies conferring sucrose resistance were transferred to D5 plates containing Cm by toothpicks. The Suc^rCm^s (r: resistant; s:

sensitive) colonies carrying either the wild type or mutant allele in the chromosome were randomly selected to perform colony PCR for screening the deletion mutants using two primer sets (sacB-F/sacB-R, 1415-uF/1415-dR). The primers (sacB-F/sacB-R) were used to verify excision of the suicide vector, while the primers (1415-uF/1415-dR) were used to screen the mutant allele in *C. salexigens*. In addition, PCR tests with an external primer 1415-test-F (anchoring upstream of integration site) and an internal primer 1415-test-R (anchoring upstream homologous region of *csal_1415* mutant allele) were used for detection of the site-specific plasmid excision event with the wild-type genomic DNA served as control. Positive colonies were further purified and confirmed by PCR and DNA sequencing. One of the positive clones was designated as *C. salexigens* 3043*Δcsal_1415*. A schematic diagram for construction of the *csal_1415* in-frame deletion mutant is shown in Fig. 2.

A similar approach was used to generate the *sarC* gene in-frame deletion mutant of *C. salexigens* ZW4-1. pMDCΔsarC was used as the allelic exchange vector. Transconjugants resulting from a single crossover event via homologous recombinant were verified with the primers sacB-F and sacB-R. To identify *ΔsarC* mutants, three primer sets were employed in colony PCR detection. Primers sacB-F and sacB-R were used to verify loss of the suicide vector, and primers sarC-uF and sarC-dR were used to verify *sarC* gene deletion in *C. salexigens*, while primers sar-test-F and sar-test-R were used for detection of the site-specific plasmid excision event. One of the positive clones was designated as *C. salexigens* 3043*ΔsarC*.

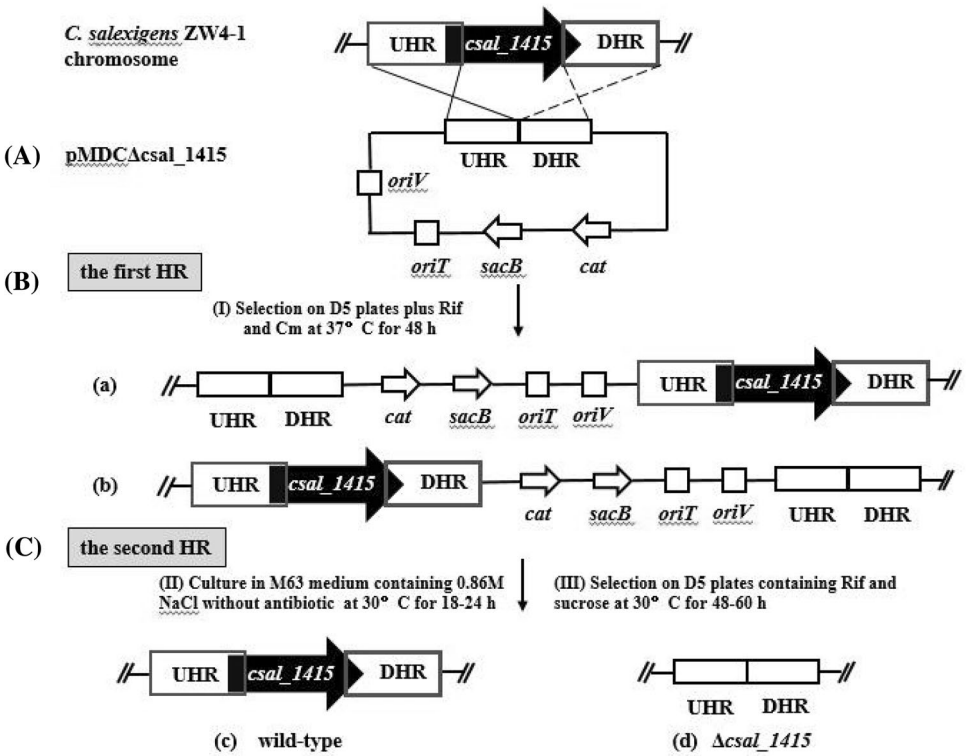

Fig. 2 Schematic diagram of allelic exchange procedures for the construction of *C. salexigens Δcsal_1415* mutant. **a** Two homologous regions (UHR and DHR, ~1 kb each) of ORF *csal_1415* was cloned into the integrative vector pMDC21, yielding pMDCΔcsal_1415. **b** The allele replacement vector pMDCΔcsal_1415 was transferred into *C. salexigens* ZW4-1 by conjugation and integrated into the target locus via one of the homologous regions (UHR or DHR) through the first homologous recombination (HR) event, resulting in Cm^r merodiploids. The first HR events are represented by *solid lines* (through the upstream homologous region, UHR) and *dashed lines* (through the downstream homologous region n, DHR). Two possible genotypes for the merodiploids are follows: *a* genotype of the merodiploid arising by the first HR through the UHR; *b* genotype of the merodiploid arising by the first HR through the DHR. **c** The merodiploid was grown in M63 medium without antibiotic pressure to induce the second crossover which resulted in the loss of the integrated plasmid, then the culture was plated on D5 plates containing sucrose with and without Cm, and incubated at 25 °C. The colonies exhibiting Suc^rCm^s phenotype were picked up and screened for the deletion mutants by colony PCR. If the second HR event occurs on the same side as the first HR event, the wild-type genotype is restored (*c*). If the second HR event occurs on the opposite side, the wild-type allele is replaced by the mutant allele (*d*)

For the construction of *csal_1415* and *sarC* double-gene in-frame deletion mutant, pMDCΔsarC was used as allele replacement vector, and *C. salexigens* 3043*Δcsal_1415* strain was used as the recipient cell in conjugation experiment. The primers used for verifying the first and second recombination events, as well as confirmation of the gene deletion, were the same as those used in construction of *C. salexigens* 3043*ΔsarC*. One of the mutant was designated as *C. salexigens* 3043*Δcsal_1415ΔsarC*.

Growth characteristics of wild type versus mutant of *C. salexigens*

To measure the growth difference between wild type and *Δcsal_1415* mutant, *C. salexigens* DSM 3043, ZW4-1, 3043*Δcsal_1415* and 3043*Δcsal_1415ΔsarC* strains were individually inoculated to D5 liquid medium, and incubated at 37 °C until early stationary phase. Then, the cells were collected by centrifugation, washed with 10 mM $MgSO_4$ for three times, and resuspended in M63 medium. Subsequently, 1% (v/v) cell suspension, calibrated with growth medium to $OD_{600} = 1.0$, were inoculated into M63 media supplemented with 5, 10, and 15% (w/v) NaCl, and the culture was incubated at 37 °C with shaking. Growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}).

To determine the ability of wild type, *ΔsarC* and *Δcsal_1415ΔsarC* mutant strains using glycine betaine, DMG, sarcosine and glycine as their sole carbon source, *C. salexigens* DSM 3043, ZW4-1, 3043*ΔsarC*, and 3043*Δcsal_1415ΔsarC* strains were streaked on M63 agar plates containing 0.86 M NaCl with glucose, glycine betaine, DMG, sarcosine or glycine as the sole carbon source, and cultivated at 37 °C for 72 h.

Results and discussion

Choosing the suitable selection markers

The method of allelic exchange between a target gene in the chromosomal DNA and a mutated allele carried by a suicide plasmid, was used to construct the unmarked deletion mutants of *C. salexigens*. The suicide plasmid normally harbored an antibiotic-resistant marker and a counterselectable marker such as *sacB*, *rpsL* or *tetAR* (Reyrat et al. 1998). The *sacB* gene from *Bacillus subtilis* encodes a levansucrase, which is lethal in most Gram-negative bacteria in the presence of sucrose (Gay et al. 1983; Reyrat et al. 1998). Salvador et al. had used *sacB* gene as the counterselection marker for screening of *C. salexigens* DSM 3043 mutants. However, a Ω cassette containing streptomycin resistance gene still existed in the mutant chromosome (Salvador et al. 2015). In order to make multiple gene deletion in *C. salexigens*, we sought to validate the suitable selection markers for unmarked allelic exchange. Plasmid pK18mobsacB (Schäfer et al. 1994) carries the pUC-based origin of replication, which makes it impossible to replicate in *C. salexigens* cells, and contains the *sacB* gene from *Bacillus subtilis* and a kanamycin resistance cassette. Previously, Grammann et al. had used pK18mobsacB as a suicide vector for the construction of *Halomonas elongata* DSM 2581 mutants impaired in biosynthesis or transport of ectoine (Grammann et al. 2002). Although *C. salexigens* is sensitive to kanamycin (100 μg ml^{-1}) at low salt concentrations (≤0.6 M NaCl), we found that the minimal inhibition concentration of this antibiotic increased by four times at elevated salt concentration (2 M NaCl). The influence of salinity on the response of various moderate halophilic bacteria to the different antimicrobials was heterogeneous, generally exhibiting three different patterns (Coronado et al. 1995). The phenomena that increasing the salt concentration in growth media may lead to a reduced inhibitory effect of some antibiotics to moderate halophiles had been reported previously (Kunte and Galinski 1995; Nieto et al. 1993), although the resistance mechanism was exactly unclear. There were some evidences indicated that several antibiotics could react with ions under high salinity, which may affect its permeability across the membrane (Piekarski et al. 2009), and the chemical changes in membrane lipids in response to the high salt concentration may alter the membrane permeability toward antibiotics (Ventosa and Nieto 2005). The resistance of *C. salexigens* ZW4-1 toward kanamycin will result in predominantly false positives clones following conjugation. Fortunately, we found that the inhibitory effect of chloramphenicol (70 μg ml^{-1}) toward *C. salexigens* was stable at 37 °C for 48 h even in the presence of 2.0 M NaCl (data not shown). Therefore, a chloramphenicol resistance cassette was PCR amplified from pACYCDuet-1 and inserted into pK18mobsacB, yielding pMDC21, which was used to select for transconjugants with plasmid integration arising from a single crossover of homologous recombination.

Generation of Δcsal_1415 mutant and phenotypic characterization of the mutant

It is well known that bacterial NhaC encodes a Na^+/H^+ antiporter, which plays an important role in maintaining cytoplasmic Na^+ homeostasis in cellular osmoregulation (Ito et al. 1997). Eight homologous genes of *nhaC* were identified in the genome of *C. salexigens* (Ates et al. 2011). To verify the validity of above-mentioned gene replacement system, ORF *csal_1415* which may code for one of the putative Na^+/H^+ antiporters was selected as the first gene for deletion. Plasmid pMDCΔcsal_1415 was used

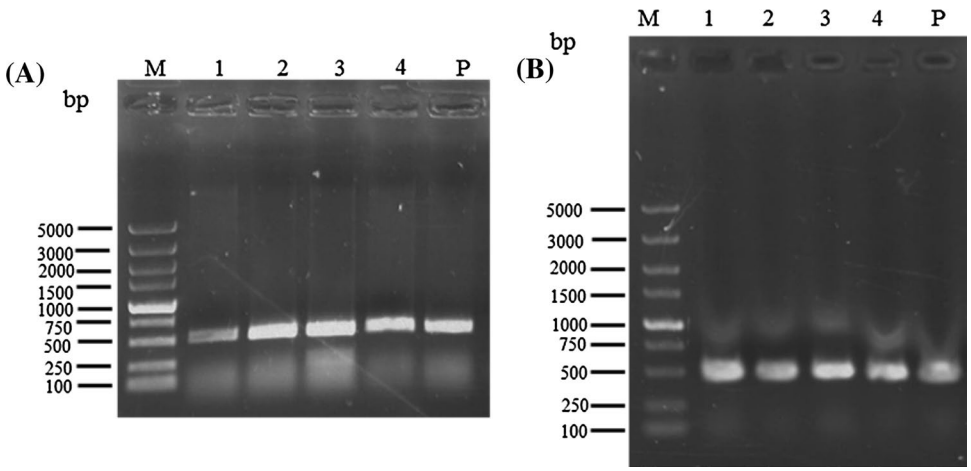

Fig. 3 Colony PCR analysis with primers sacB-F and sacB-R for verifying the integration of allelic exchange vector into the chromosome of *C. salexigens* ZW4-1 by the first homologous recombination event. **a** Verification of the integration of pMDCΔcsal_1415. **b** Verification of the integration of pMDCΔsarC. *Lane M* DL 5000 DNA Marker (TaKaRa); *lane 1–4* chloramphenicol resistant colonies; *lane P* positive control: *E. coli* 5α (pK18mobsacB)

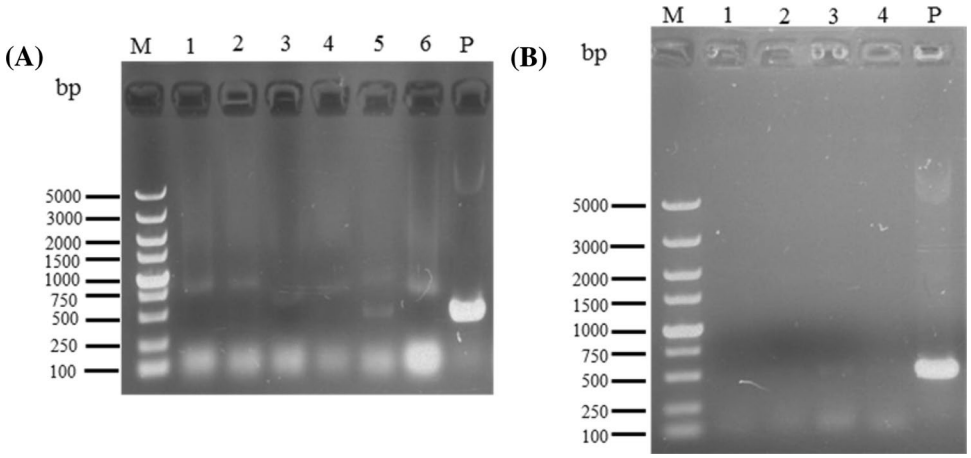

Fig. 4 Colony PCR analysis with primers sacB-F and sacB-R for verifying the loss of the integrated plasmid from the chromosome of *C. salexigens* ZW4-1 by the second homologous recombination event. **a** Verifying the loss of the pMDCΔcsal_1415 backbone; **b** verifying the loss of the pMDCΔsarC backbone. *Lane M* DL 5000 DNA Marker (TaKaRa); *lane 1–6* sucrose-resistant, chloramphenicol-sensitive colonies; *lane P* positive control: *E. coli* 5α (pK18mobsacB)

to introduce the mutant allele of *csal_1415* lacking the internal 1116-bp DNA fragment of wild-type allele into *C. salexigens* ZW4-1 chromosome. Of the colonies that grew on chloramphenicol selective plates, a 507-bp fragment was amplified by colony PCR using primers sacB-F and sacB-R from all 20 colonies randomly selected, indicating that plasmid integration event had occurred (Fig. 3a). In the plasmid excision step, 100 colonies growing on sucrose-containing plates were randomly selected and transferred to D5 plates with or without chloramphenicol by toothpicks. Among them, 32% of clones showed Suc^rCm^r phenotype, while the remaining showed Suc^rCm^s phenotype. Colony PCR with the primers sacB-F and sacB-R indicated that all Suc^rCm^s clones lost the suicide vector (Fig. 4a), while the vector backbone was still maintained in the chromosome of the Suc^rCm^r clones, but *sacB* gene was inactive (data not shown). Screening for mutants among the Suc^rCm^s colonies was performed by PCR using primers 1415-uF and1415-dR, and amplifying 3006 bp from the wild type and 1890 bp from the mutant (Fig. 5a). In addition, primers 1415-test-F and 1415-test-R were used to verify that

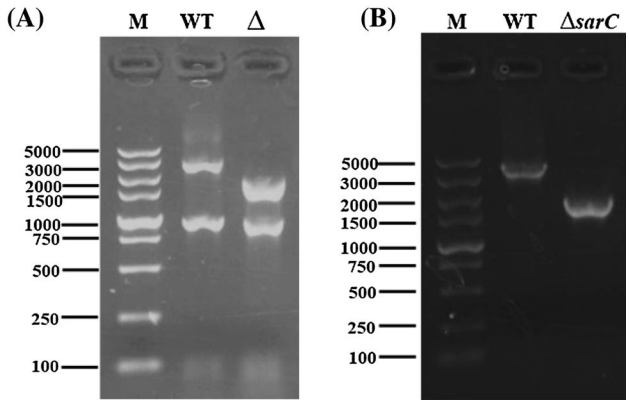

Fig. 5 Verification of the deletion mutants by colony PCR. **a** PCR products using the primers 1415-uF and 1415-dR were separated by agarose electrophoresis resulting in a band at 3006 bp for the wild type (WT) and at 1890 bp for the *Δcsal_1415* mutant (Δ). As for the additional band at approximate 1 kb, DNA sequencing results indicated that it consisted of the entire downstream homologous region of Csal_1415 due to the non-specific binding of primer 1415-uR. **b** PCR products using the primers sarC-uF and sarC-dR resulting in a band at 3943 bp for the wild type (WT) and at 1899 bp for the *ΔsarC* mutant. *Lane M* DL 5000 DNA marker (TaKaRa)

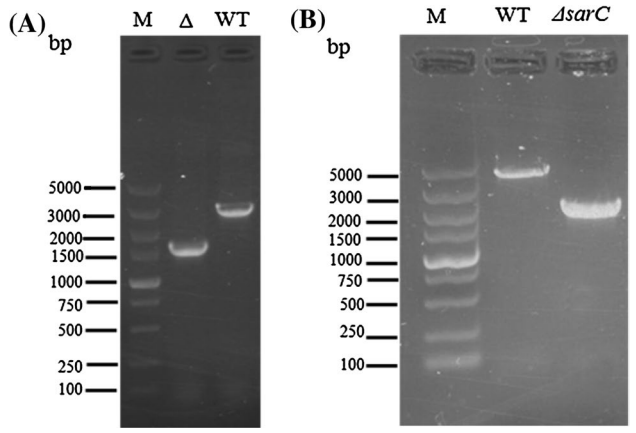

Fig. 6 Verification of the allelic exchange occurred at the desired locus by colony PCR. **a** PCR products using the primers 1415-test-F and 1415-test-R were separated by agarose electrophoresis resulting in a band at 2746 bp for the wild type (WT) and at 1630 bp for the *Δcsal_1415* mutant (Δ). **b** PCR products using the primers sarC-test-F and sarC-test-R resulting in a band at 4128 bp for the wild type (WT) and at 2159 bp for the *ΔsarC* mutant. *Lane M* DL 5000 DNA marker (TaKaRa)

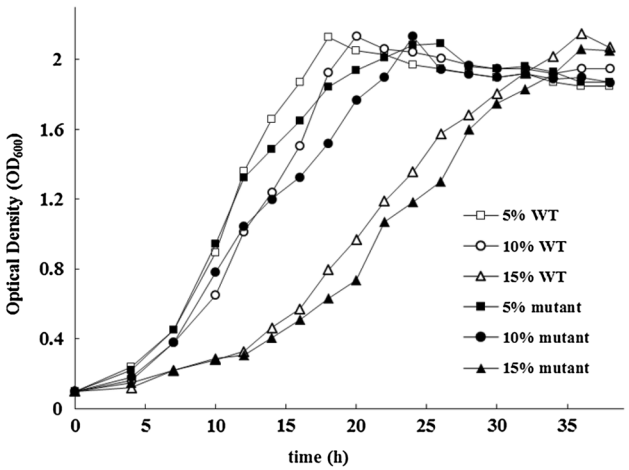

Fig. 7 Growth characteristics of *C. salexigens* ZW4-1 and 3043*Δcsal_1415* strains. Both strains were grown at 37 °C in M63 medium containing different NaCl concentrations with shaking. The samples were withdrawn periodically to measure the optical density at 600 nm (OD_{600})

the second homologous recombination event occurred at the desired locus. The *Δcsal_1415* mutant could generate the predicted 1630-bp amplicon, while the wild-type strain produces 2746-bp amplicon (Fig. 6a). In fact, none of the mutants were proved to be off-targeted, and the sequencing of PCR products also confirmed that the deletion mutants were successfully obtained. Of the 251 analyzed Suc^rCm^s colonies, 2 had the desired *Δcsal_1415* genotype by PCR and DNA fragment sequencing, designated as 3043*Δcsal_1415* strain.

To test the effect of *csal_1415* deletion on cellular osmoadaptation, the growth of wild type and *Δcsal_1415* mutant in M63 medium containing different NaCl concentrations was determined by OD_{600}. Our results showed that the *Δcsal_1415* mutant did not exhibit a salt-sensitive phenotype, and its salinity upper limit was unchanged as compared with that of wild-type strain. As shown in Fig. 7, the *Δcsal_1415* mutant showed similar growth curves with those of the wild type in M63 media containing different NaCl concentrations, suggesting that ORF *csal_1415* is not directly involved in osmoregulation in the presence of various NaCl concentrations, and the exactly role of this gene in *C. salexigens* remains to be further elucidated.

Generation of *ΔsarC* mutant and phenotypic characterization of the mutant

To further validate the method, *sarC* gene deletion was performed. Based on amino acid sequence homology analysis, *csal_0998*, *csal_0999*, *csal_1000* and *csal_1001* code for four different subunits (β, δ, α, and γ, respectively) of heterotetrameric sarcosine oxidase (SarC), which is involved in glycine betaine catabolism and catalyzes the conversion of sarcosine to glycine (Ida et al. 2005). To introduce the deleted *sarC* locus into the chromosome of *C. salexigens* ZW4-1, plasmid pMDCΔsarC was used as the allele replacement vector. In the plasmid integration step, all of 20 randomly selected Cm^r colonies contained the suicide vector integrated in the chromosomal DNA as detected by colony PCR with primers sacB-F and sacB-R (Fig. 3b). In the plasmid excision step, 36% (36/100) of Suc^r colonies exhibited Suc^rCm^r phenotype, while the remaining showed Suc^rCm^s phenotype. The Suc^rCm^s colonies were further analyzed by colony PCR with primers to check for the suicide vector (primers sacB-F and sacB-R) and mutant allele (primers sarC-uF and sarC-dR). All of the Suc^rCm^s clones tested lost the vector backbone as verified by PCR with primes sacB-F and sacB-R (Fig. 4b), and the expected in-frame deletion produced a 1.9-kb amplicon, while the wild type yielded a 3.9-kb amplicon with primers sarC-uF and sarC-dR (Fig. 5b). To further verify that the mutant allele of *sarC* gene was located at the desired locus, colony PCR was performed with primers sarC-test-F and sarC-test-R, resulting in a 2.1-kb fragment which is consistent with what we predicted (Fig. 6b). Of the 371 Suc^rCm^s colonies analyzed, two colonies were confirmed to be genuine mutants by PCR and DNA fragment sequencing.

To verify the biological effect of *ΔsarC* deletion mutation in *C. salexigens*, the phenotype arising from the deletion of *sarC* was analyzed. Since the sarcosine oxidase SarC is involved in glycine betaine catabolism, the wild type and *ΔsarC* mutant were tested for their abilities to utilize glycine betaine and DMG as the sole carbon source.

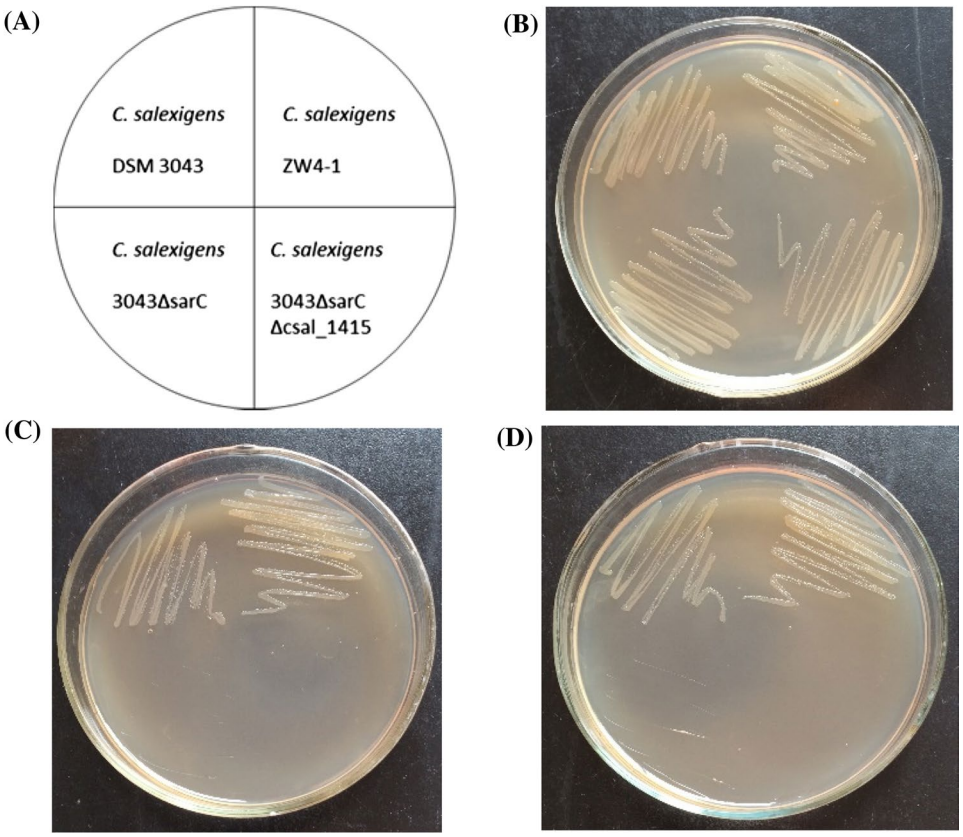

Fig. 8 Carbon utilization tests for *C. salexigens* wild-type and deletion mutants. **a** Diagram of four quadrants on a plate in which the strains were streaked. *C. salexigens* DSM 3043, ZW4-1, 3043*ΔsarC*, and 3043*ΔsarCΔcsal_1415* strains were streaked on M63 agar plates containing rifampicin and 0.86 M NaCl with glucose (**b**), glycine betaine (**c**) or dimethylglycine (**d**) as the sole carbon source, and incubated at 37 °C for 48–60 h

As shown in Fig. 8, when grown in M63 minimal medium supplemented with Rif and 0.86 M NaCl, strain ZW4-1 could utilize glucose, glycine betaine and dimethylglycine as the sole carbon sources, while 3043*ΔsarC* strain could use glucose as the sole carbon sources, but not for glycine betaine and dimethylglycine. The phenotypes of wild-type and mutant strains are consistent with the predicted function of *sarC* gene in cellular metabolism. Interestingly, we found that neither wild type nor *ΔsarC* mutant strain could use sarcosine or glycine as the sole carbon source. The reason may appear to be lacking the appropriate transporters located on cellular membrane to transport these substrates into the cells.

Generation of *Δcsal_1415ΔsarC* mutant and phenotypic characterization of the mutant

One of the advantages for employing unmarked mutations is that multiple modifications can be made in a single strain using the same selection system. To test the feasibility for construction of multiple gene deletion mutant with this system in *C. salexigens*, *Δcsal_1415ΔsarC* double-gene deletion mutant was constructed. Conjugation was carried out between *E. coli* S17-1 λpir carrying pMDCΔcsal_1415 and *C. salexigens* 3043*ΔsarC* strain. To identify double-gene in-frame deletion mutants, colony PCR was carried out with primers the same as those used in construction of *C. salexigens* 3043*Δcsal_1415* strain (data not shown). Among the 167 randomly selected Suc^rCm^s colonies, 2 colonies were confirmed to have the desired deletion by PCR and DNA fragment sequencing.

The growth curve in minimal medium M63 under different NaCl concentrations and the ability to utilize substrates as sole carbon source for *C. salexigens* 3043*Δcsal_1415ΔsarC* were determined under the same conditions as those for 3043*Δcsal_1415* and 3043*ΔsarC* strains. As shown in Fig. 8, strain 3043*Δcsal_1415ΔsarC* could not use glycine betaine and dimethylglycine as the sole carbon source, the same as that for strain 3043*ΔsarC*. Based on comparison of cell growth curves, there is no significant difference among the WT, 3043*Δcsal_1415*, and 3043*Δcsal_1415ΔsarC* strains when grown in M63 medium with glucose as the sole carbon source (data not shown).

Incubation at 25 °C drastically decreases the proportion of spontaneous Suc^r colonies

Although the *sacB* gene from *B. subtilis* has been used successfully as a counterselectable marker to generate unmarked mutants in many Gram-negative bacteria, one major problem using *sacB* gene is the occurrence of

spontaneous sucrose resistance (SSR) colonies during the screening process since *sacB* gene was inactivated by either a mutation, or an IS element as previously described in other bacteria (Cai and Wolk 1990; Gay et al. 1985; Jager et al. 1995; van Aartsen and Rajakumar 2011), rather than lost as in authentic double recombinants. The SSR colonies still retain the integrative vector backbone in chromosome and show resistance to antibiotics, and have been observed at frequencies of 2–50% in other bacteria (Wu and Kaiser 1996). Blomfield et al. found that incubation at 30 °C on negative selection media could markedly increase sucrose sensitivity as opposed to 37 °C, and decreases the proportion of pseudo-double recombinants among the Suc^r colonies (Blomfield et al. 1991). To further investigate the effect of incubation temperature on the occurrence of SSR colonies, the merodiploid in which pMDCΔsarC was integrated into the chromosome of *C. salexigens* ZW4-1, were inoculated in D5 medium without adding antibiotic, and incubated at 37, 30, and 25 °C, respectively. Subsequently, the cultures were individually spread on sucrose-containing plates and incubated at the same temperatures as those before. The ratios of SSR colonies among the Suc^r colonies were determined to be 51 ± 9, 27 ± 10, and 1 ± 1%, respectively, indicating that growth of the merodiploid and *sacB* counterselection at 25 °C dramatically decreases the observed SSR rate as compared with those at 30 or 37 °C, and minimizes the number of clones to be analyzed by colony PCR.

Growth of the merodiploids in minimal medium instead of complex medium increases the recovery rate of deletion mutants

Although incubation at 25 °C in the couterselection process could significantly decrease the proportion of SSR colonies, the recovery rate of the desired mutants was only about 0.7–1.3% in *C. salexigens* even after the false-positive recombinants were excluded, which was not consistent with our expectation. Theoretically, if the targeted gene is not vital, the second recombination leads to either wild-type allele restoration or to allelic substitution at a 1:1 ratio when the two homologous regions flanking the target gene to be deleted were the similar sizes. The overall poor recovery of the desired mutants prompted us to test whether the presence of NaCl in the counterselection medium would affect the recovery efficiency of desired recombinants, since the presence of sodium chloride in the selective medium had been reported to be another factor that may influence the counterselection efficiency with *sacB* (Blomfield et al. 1991). As a moderate halophile, *C. salexigens* DSM 3043 requires at least 0.5 M NaCl for growth in M63 medium, and its growth can be promoted combined with other ions (O'Connor and Csonka 2003). To avoid the negative effect of NaCl on the recovery efficiency, we tried to replace NaCl with other salts in media that could support the growth of *C. salexigens* and found that ZW4-1 strain could grow in YET medium supplemented with $MgSO_4$ (0.35–2.3 M) without adding NaCl. Therefore, three different kinds of media: YET medium plus 0.86 M NaCl, YET medium plus 0.42 M $MgSO_4$, and M63 medium plus 0.86 M NaCl, were used for the growth of the merodiploids in which pMDCΔsarC was integrated into the chromosome of *C. salexigens* ZW4-1 in the absence of antibiotic and incubated at 25 °C with shaking for 40 h. Subsequently, the cultures were individually plated on YET agar plates containing sucrose and 0.42 M $MgSO_4$, and incubated at 25 °C until the colonies were visible. However, our results indicated that replacement of NaCl with $MgSO_4$ had no effect on improving the recovery efficiency of deletion mutants in *C. salexigens* (data not shown). Unexpectedly, the recovery rate of desired recombinants was dramatically increased in the case of growing in M63 medium plus 0.86 NaCl compared with those formerly grown in YET medium plus 0.86 M NaCl or YET medium plus 0.42 M $MgSO_4$. Based on the result of colony PCR screening with the primers sarC-uF and sarC-dR, 32% (16/50) of Suc^rCm^s colonies formerly grown in M63 medium plus 0.86 M NaCl were verified to be genuine double crossover mutants, while only 4% (2/50) and 2% (1/50) of colonies formerly grown in YET medium with NaCl and YET medium with $MgSO_4$ carried the *sarC* deletion, which indicated that the medium composition, not just confined to NaCl, had an obvious influence on the recovery rate of deletion mutants, although the exact reasons for this phenomenon remain unknown. To further investigate the role of sucrose-containing media on the recovery rate of the desired recombinants, the culture formerly grown in M63 medium plus 0.86 M NaCl was plated on M63 plates containing 0.86 M NaCl and sucrose, D5 plates containing sucrose, and YET plates containing 0.42 M MgSO4 and sucrose, respectively, but there was no obvious effect on increasing the recovery rate of desired recombinants (data not shown). Since ZW4-1 strain reached the highest growth rate when cultured in D5 medium, D5 medium supplemented with sucrose was ultimately employed as the negative selection medium.

Conclusions

In this report, a markerless gene deletion system via allelic exchange was first successfully developed in *C. salexigens*, which can be used for functional gene analysis as well as for other applications, such as multiple point mutations or insertion in *C. salexigens* chromosome DNA, and it expands the genetic tools for *Chromohalobacter*. Critically, our results show that growth of the merodiploids and

sucrose screening at 25 °C is more effective to decrease the occurrence of spontaneous sucrose resistance colonies than at higher temperature with *sacB* gene as the couterselection marker in *C. salexigens*, and growth of the merodiploids in mineral salt medium instead of complex medium is critical to dramatically increase the recovery rate of deletion mutants. These new findings not only are applicable for the construction of deletion mutants in *C. salexigens*, but also may shed light on allelic exchange with *sacB* gene as the counterselectable marker in the other halophiles or marine microorganisms, which need the necessary presence of NaCl for the normal growth.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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