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Yanhong Wang

Heilongjiang Bayi Agricultural University

Jiahui Chen

Heilongjiang Bayi Agricultural University

Beibei Xu

Heilongjiang Bayi Agricultural University

Amro Abdelazez

amorbiotic@yahoo.com

Linyi University

Heba Abdel-motaal

Linyi University

YiTong Feng

Heilongjiang Bayi Agricultural University

Yaru Zhu

Heilongjiang Bayi Agricultural University

Wenzhao Wang

Heilongjiang Bayi Agricultural University

Jingxuan Pan

Heilongjiang Bayi Agricultural University

Ming Yang

Heilongjiang Bayi Agricultural University

Wang Hongyu

Heilongjiang Bayi Agricultural University

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Tricarboxylic Acid Cycle Genes *sucA* and *sucC* Confer Salt-Alkaline Tolerance in *Enterobacter kobei* by Enhancing Energy Metabolism and Carbon Source Utilization

Yanhong Wang ^{a,†,*}, Jiahui Chen^{a,†}, Beibei Xu ^{a,†}, Amro Abdelazez ^{b,c,*}, Heba Abdel-motaal ^{b,d,*}, YiTong Feng^a, Yaru Zhu ^a, Wenzhao Wang ^a, Jingxuan Pan ^a, Ming Yang ^a, and Wang Hongyu ^a

^a College of Life Science and Biotechnology, Heilongjiang Bayi Agricultural University, Heilongjiang Provincial Key Laboratory of Environmental Microbiology and Recycling of Argo-Waste in Cold Region, Daqing 163319, China

^b College of Agriculture and Forestry, Linyi University, Linyi, 276005, Shandong, China

^c Department of Dairy Microbiology, Animal Production Research Institute, Agriculture Research Centre, Dokki, 12618, Giza, Egypt

^d Department of Microbiology, Soils, Water, and Environment Research Institute, Agriculture Research Centre, 12618, Giza, Egypt

*Correspondence: Amro Abdelazez: amorbiotic@yahoo.com; Heba Abdel-motaal: heba@lyu.edu.cn; Yanhong Wang: wylh_03@126.com

† These authors contributed equally to this work.

Abstract

Soil salinization threatens global agriculture, necessitating the discovery of robust microbial resources for bioremediation. Here, we characterized *Enterobacter kobei* P3, a halotolerant bacterium isolated from Egyptian saline-alkaline soils. Phenotypic and genomic analyses revealed broad environmental adaptability, with optimal growth at 30°C, 3% NaCl, and pH 8.5. Transcriptomic profiling under saline-alkaline stress identified the tricarboxylic acid (TCA) cycle as a critical response pathway, with *sucA* and *sucC* emerging as significantly upregulated central hub genes. Functional complementation in *Escherichia coli* demonstrated that co-expressing *sucA* and *sucC* synergistically enhanced host survival under high salinity (up to 1.0 M NaCl) and alkaline pH (up to 9.5), significantly outperforming single-gene expressions and wild-type controls. Conversely, a *sucC* knockout mutant exhibited severe growth inhibition. Metabolic profiling confirmed that *sucAC* overexpression optimized carbon flux by accelerating reducing sugar consumption and elevating intracellular succinyl-CoA levels under stress. These findings elucidate a molecular mechanism whereby *sucA* and *sucC* confer saline-alkaline tolerance through TCA cycle-mediated energy supply and carbon utilization. Ultimately, this study identifies the *sucAC* genes as valuable genetic targets for engineering stress-resilient microbes to bioremediate degraded saline-alkaline soils.

Keywords: *Enterobacter kobei*; Salt-alkaline stress; Transcriptome; *sucA*; *sucC*; Tricarboxylic acid cycle; Energy metabolism; Soil bioremediation

1. Introduction

Soil salinization has emerged as one of the most pervasive forms of land degradation, currently affecting approximately 1 billion hectares of global arable land and threatening the food security of nearly 50% of the world's population by mid-century (1, 3, 6). The accumulation of excess soluble salts and the dominance of sodium ions under high-pH conditions collectively disrupt soil structure, impede water infiltration, and impose severe ionic and osmotic stress on crops, ultimately leading to substantial yield penalties (4, 5). Furthermore, salinization reduces soil microbial diversity and activity, degrades soil aggregate structure, and accelerates vegetation degradation (24). With an increasing population and decreasing arable land resources, the remediation of saline-alkali soils has become a critical issue for global food security (38).

Traditional physicochemical remediation strategies, including leaching, gypsum application, and the use of synthetic amendments, often prove economically prohibitive and environmentally unsustainable at scale, particularly for smallholder farming systems in arid and semi-arid regions. Consequently, biological approaches harnessing salt-tolerant plant growth-promoting rhizobacteria (PGPR) and extremophilic microbes have garnered considerable attention as cost-effective, eco-friendly alternatives for the restoration of saline alkaline ecosystems (18, 33, 1).

Bacterial adaptation to saline-alkaline environments requires a coordinated physiological response to maintain osmotic balance, ion homeostasis, and macromolecular stability. To survive severe osmotic and pH stress, halotolerant microorganisms employ sophisticated adaptive strategies. These include the accumulation of compatible solutes (e.g., glycine betaine, ectoine, and trehalose) to preserve cellular hydration, the activation of specialized ion transporters (e.g., Na^+/H^+ antiporters) to extrude toxic ions, membrane lipid remodeling, and the upregulation of antioxidant systems to mitigate oxidative damage (8-10, 30, 39)

Within this context, the genus *Enterobacter* stands out for its remarkable metabolic versatility and environmental resilience. Salt-tolerant *Enterobacterales* have garnered significant attention as plant-growth-promoting bacteria (PGPB) capable of thriving in saline soils while enhancing plant nutrition and stress tolerance (14, 25). As modern biotechnologies increasingly leverage microbial gene resources and molecular markers to develop salt-tolerant crop varieties (20, 22), deciphering the specific molecular and metabolic mechanisms of indigenous halotolerant *Enterobacter* strains is essential. Unlocking these adaptive traits not only enriches the microbial gene pool for saline-alkali soil bioremediation but also provides critical genetic resources for sustainable agricultural management in extreme environments (19).

Central carbon metabolism, particularly the tricarboxylic acid (TCA) cycle, is essential for meeting the energetic and biosynthetic demands of saline-alkaline stress (39). However, the genetic determinants governing TCA-mediated tolerance in enteric bacteria remain poorly understood. Although *sucA* and *sucC* encode rate-limiting TCA cycle enzymes, their synergistic role in enhancing carbon utilization and energy transduction under saline-alkaline conditions remains unexplored, as prior single-gene studies overlook critical metabolic flux coordination. To address this, we isolated the moderately halotolerant *Enterobacter kobei* P3 from Egyptian saline-alkaline soils. By integrating transcriptomics with functional genetics in *Escherichia coli*, we identified *sucA* and *sucC* as pivotal stress-response hubs. We hypothesize that their co-expression optimizes TCA cycle flux, ensuring sustained ATP production and conferring superior stress tolerance compared to single-gene interventions. Ultimately, this work expands the microbial stress-

tolerance gene repertoire and provides a translational framework for engineering robust bacterial inoculants for soil bioremediation.

2. Materials and Methods

2.1. Bacterial Strain Isolation, Plasmids, and Culture Conditions

The bacterial strain designated P3 was isolated from surface agricultural soil (0–20 cm depth) collected in August 2022 from the South El-Hosynia Research Farm Station, Sharkia Governorate, Egypt (31°00'59.2" N, 32°04'17.2" E). *Escherichia coli* KNabc was generously provided by Professor Juquan Jiang (Faculty of Life Sciences, Northeast Agricultural University, Harbin, China). All bacterial strains and plasmids utilized in this study are detailed in Table S1.

Luria-Bertani (LB) medium and M9 minimal medium were employed for strain cultivation. LB medium comprised 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1.0% (w/v) sodium chloride. M9 minimal medium contained 0.678% (w/v) disodium hydrogen phosphate, 0.3% (w/v) potassium dihydrogen phosphate, 0.1% (w/v) ammonium chloride, 0.05% (w/v) sodium chloride, 0.024% (w/v) magnesium sulfate, 0.0011% (w/v) calcium chloride, and 0.4% (w/v) glucose, with glucose serving as the primary carbon source to evaluate sugar metabolism and carbon source utilization under saline-alkali stress conditions. All cultures were incubated at 37 °C with shaking at 160 rpm. Antibiotics kanamycin, chloramphenicol, and ampicillin (Bio-sharp, Hefei, China) were added to media at final concentrations of 50 µg/mL, 25 µg/mL, and 50 µg/mL, respectively, as required.

2.2. Phenotypic Characterization of Salt-Alkaline Tolerance

Bacterial isolates were identified based on morphological and biochemical characteristics, including cell morphology, Gram staining reaction, and colony morphology, following standard microbiological protocols. Strain P3 was pre-cultured in LB broth at 30 °C for 12 h prior to phenotypic assays.

To evaluate temperature tolerance, a 1% (v/v) inoculum of the pre-culture was transferred into 100 mL of fresh LB medium and incubated at varying temperatures (10, 15, 20, 25, 30, 35, and 40 °C) in a temperature-controlled shaking incubator (SHZ-82, Jiangsu Jintan Liangyou, China). Bacterial growth was monitored spectrophotometrically.

For salt and alkali tolerance assays, LB media were prepared with NaCl concentrations of 0.75, 1.0, 1.25, and 1.5 M, and adjusted to pH values of 8.5, 9.0, 9.5, and 10.0, respectively. Following autoclaving, each treatment was inoculated with 1% (v/v) pre-culture in triplicate. Cultures were incubated at 30 °C with shaking at 140 rpm in an orbital shaker (ZHWY-200B, Shanghai Zhicheng Analytical Instrument, China). Growth was evaluated by measuring optical density at 600 nm (OD₆₀₀) using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 8-h intervals. Monitoring continued until cultures reached the decline phase, and growth curves were plotted accordingly.

2.3. Molecular Characterization and Phylogenetic Analysis

Genomic DNA was extracted from the target strain using the standard phenol–chloroform method (12). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal bacterial

primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Thermal cycling conditions consisted of initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 90 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min (Table S2).

PCR amplicons were resolved by electrophoresis on 1.0% (w/v) agarose gels (Sigma-Aldrich, St. Louis, MO, USA) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at a constant voltage of 100 V for 1 h. Gels were stained with ethidium bromide (0.2 µg/mL) for 15 min and visualized under UV transillumination (Bio-Rad, Hercules, CA, USA). A 100-bp DNA ladder (Gibco-BRL, Grand Island, NY, USA) served as the molecular weight standard.

The purified PCR product was sequenced by BGI Genomics Co., Ltd. (Shenzhen, China). The resulting 16S rRNA gene sequence was queried against the NCBI GenBank database using the BLASTn algorithm for taxonomic identification. Multiple sequence alignment was performed using ClustalW, and a phylogenetic tree was reconstructed via the neighbor-joining method implemented in MEGA 11. The complete genome sequence has been deposited in the National Microbiology Data Center under accession number NMDC60214231.

2.4. Transcriptome sequencing analysis

Enterobacter kobei P3 was activated on LB agar for 24 h, and a single colony was inoculated into LB broth under three salt-alkali stress conditions: low (X1; natural pH, 0.25 M NaCl), moderate (X2; pH 8.5, 0.75 M NaCl), and high (X3; pH 9.0, 1.25 M NaCl). Cultures were incubated at 30 °C with shaking until mid-exponential phase ($OD_{600} \approx 1.0$). Cells were harvested by centrifugation (8,000 × g, 10 min, 4 °C), flash-frozen in liquid nitrogen, and stored at -80 °C. Three independent biological replicates were prepared per treatment.

Total RNA extraction, rRNA depletion, library preparation, and sequencing were performed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Following RNA-quality assessment, rRNA-depleted RNA was fragmented and reverse-transcribed using random primers. The resulting cDNA underwent end repair, 3' adenylation, adapter ligation, and PCR amplification. Libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to generate 150-bp paired-end reads.

Raw reads were quality-filtered using Trimmomatic v0.39, and clean reads were mapped to the complete *E. kobei* P3 genome using HISAT2 v2.2.1. Gene-level read counts were analyzed using DESeq2 v1.34.0 for pairwise comparisons of (X2 vs. X1); (X3 vs. X2); and (X3 vs. X1). Genes with $\log_2$ (fold change) ≥ 1 and adjusted $P < 0.05$ were considered differentially expressed genes (DEGs). Functional annotation and enrichment analyses were performed using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to characterize enriched biological processes, molecular functions, cellular components, and metabolic pathways.

2.5. Bioinformatics Analysis of *SucA* and *SucC* Proteins

The nucleotide sequences of the *sucA* and *sucC* genes from *E. coli* K-12 MG1655 were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov>), and their corresponding translated amino acid sequences

were obtained from the UniProt database (<https://www.uniprot.org>). Physicochemical properties of the encoded proteins including molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY) were analyzed using ProtParam (<https://web.expasy.org/protparam>). Protein domain architecture and putative subcellular localization were predicted using InterPro (<https://www.ebi.ac.uk/interpro>). Hydrophilicity/hydrophobicity profiles were generated using ProtScale (<https://web.expasy.org/protscale>). Secondary structure elements were predicted using SOPMA (<https://www.expasy.org/search/SOPMA>), and transmembrane topologies were analyzed using DeepTMHMM (<https://dtu.biolib.com/DeepTMHMM>). Collectively, these *in silico* analyses provided a comprehensive structural and functional baseline for the *SucA* and *SucC* proteins to guide subsequent experimental validation.

2.6. Construction of Expression Vectors and Physiological Activity Validation

2.6.1. Seamless Cloning and Recombinant Plasmid Construction

Seamless cloning and assembly were performed using the pEAS-Basic Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China). The target fragments of the *sucA* and *sucC* genes were amplified from *Enterobacter kobei* P3 genomic DNA using primers containing homologous arm sequences (underlined) designed with Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA).

The pET-28a vector (Novagen, Merck KGaA, Darmstadt, Germany) was linearized by double restriction enzyme digestion with *NdeI* and *XhoI* (New England Biolabs, Ipswich, MA, USA). PCR products and linearized vector were gel-purified using a Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA), verified by agarose gel electrophoresis, and quantified spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA).

The validated PCR products (inserts) and linearized vector (5–100 ng) were mixed at a 1:2 molar ratio (vector: insert) in a 10 µL reaction containing 5 µL of 2× Basic Assembly Mix (with dye) and nuclease-free water to volume. The mixture was incubated at 50 °C for 5–15 min to assemble the recombinant plasmids pET-*sucA* (containing *sucA*), pET-*sucC* (containing *sucC*), and pET-*sucAC* (containing both *sucA* and *sucC* in a bicistronic configuration). The ligation products were transformed into competent *E. coli* KNabc cells by heat-shock transformation (42 °C for 45 s, followed by 2 min on ice).

Single colonies were verified by colony PCR using T7 promoter and T7 terminator primers. Positive clones were inoculated into LBK medium (LB medium supplemented with 50 mM KCl) containing 50 µg/mL ampicillin and cultured overnight at 37 °C with shaking (220 rpm). Recombinant plasmids were confirmed by agarose gel electrophoresis and double restriction enzyme digestion (*NdeI/XhoI*). Correctly verified plasmids were sent to BGI Genomics (Shenzhen, China) for Sanger sequencing to confirm sequence fidelity.

2.6.2. Salt Tolerance Assay of Recombinant Strains

The recombinant strains KNabc/pET-*sucA*, KNabc/pET-*sucC*, and KNabc/pET-*sucAC* were evaluated for salt tolerance, with KNabc/pET-28a (empty vector) serving as the negative control. Overnight cultures were diluted to an initial OD₆₀₀ of 0.1 in fresh LBK medium (pH 7.0) supplemented with NaCl at final concentrations of 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 M. Cultures were incubated at 30 °C with shaking (200

rpm), and cell growth was monitored by measuring OD₆₀₀ at 2-h intervals over 48 h using a microplate reader (BioTek ELx808, Winooski, VT, USA). All assays were performed in triplicate.

2.6.3. pH Tolerance Assay

To evaluate the effect of pH on cell growth, overnight cultures were diluted to an initial OD₆₀₀ of 0.1 in fresh LBK medium containing 50 mM NaCl adjusted to pH values of 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 using 1 M HCl or 1 M NaOH. Cultures were incubated at 30 °C with shaking (200 rpm), and OD₆₀₀ was measured at intervals over 48 h as described above. Experiments were performed in triplicate.

2.6.4. Overexpression and Quantitative Real-Time PCR Analysis

The recombinant plasmid pET-*sucAC* was transformed into *E. coli* BL21(DE3) competent cells (TransGen Biotech) by heat-shock transformation to generate the overexpression strain *E. coli*/pET-*sucAC*. Single colonies were selected on LB agar containing 50 µg/mL ampicillin and verified by colony PCR.

For gene expression analysis, *E. coli*/pET-*sucAC* and control (*E. coli*/pET-28a) were cultured in LB medium containing 50 µg/mL ampicillin at 37 °C until OD₆₀₀ reached 0.6–0.8. Expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, followed by incubation at 25 °C for 6 h with shaking (200 rpm). Total RNA was extracted using the RNeasy Pure Bacteria Kit (Qiagen Biotech, Beijing, China) according to the manufacturer's instructions. RNA concentration and purity were assessed spectrophotometrically (A_{260}/A_{280} ratio ≥ 1.8). cDNA was synthesized from 1 µg of total RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio, Kusatsu, Japan).

Quantitative real-time PCR (qRT-PCR) was performed using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio) on a LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland). Gene-specific primers for *sucA* and *sucC* were designed using Primer3Plus (<https://www.primer3plus.com/>). The 16S rRNA gene served as the endogenous reference control. The thermal cycling protocol consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Melt curve analysis (65–95 °C) was performed to confirm amplicon specificity. Relative gene expression was calculated using the 2^{−ΔΔCt} method, and data are presented as fold-change relative to the control strain. All reactions were performed in technical triplicate using three independent biological replicates.

2.7. Knockout of the *sucC* Gene

The knockout of the *sucC* gene was achieved using the λ-Red homologous recombination system, a widely adopted method for generating gene-deficient strains (Fig. 1). The strain Δ*sucC* was constructed to create a targeted deletion of the *sucC* gene. The pKD46 plasmid, which expresses three critical enzymes (Exo, Bet, and Gam) essential for homologous recombination, was utilized in this process and was induced by L-arabinose to facilitate efficient recombination events.

For gene knockout primer design, the forward primer sequence was: 5'-acaagcgatgcctgatgtgacgtttaacgtgtcttatcaggcctacgggtgaccgacaatgcccggaagcgatacgaaatattcggtctacggttataaagataacgattactgaaggatTTAACGGCTGACATGGGAATTAGC-3, and the reverse primer sequence was: 5'-

gcctttacctgggggttacgccgccaaccatttttagtgccgtatgcaatggcctgttctgagtggaaagtccctggctaccggtaaagccctggcagataac
cttggtgtttttatcgatTTGAGCGATTGTGTAGGCTGGAG-3'. The underlined regions indicate homology arms
corresponding to the *sucC* gene. Each primer contained a chloramphenicol resistance cassette (cat),
enabling targeted integration of the knockout construct into the bacterial genome via homologous
recombination.

E. coli cells harboring the pKD46 plasmid were cultured at 30 °C to logarithmic growth phase (OD₆₀₀ = 0.4–
0.6). L-Arabinose (final concentration 10 mM) was then added to induce expression of the recombination
enzymes for 1 h. The purified targeting fragment was subsequently electroporated into the induced
electrocompetent cells using a Bio-Rad electroporation system (Bio-Rad, Hercules, CA, USA) at 2.5 kV, 25
µF, and 200 Ω. Following electroporation, cells were incubated at 37 °C for 2 h in SOC medium to allow
recovery and expression of the antibiotic resistance gene. Positive clones with successful integration of the
chloramphenicol resistance gene into the *sucC* locus were selected on LB agar plates containing 25 µg/mL
chloramphenicol. Clones were cultured in chloramphenicol-supplemented medium to maintain selection
pressure and finally confirmed by Sanger sequencing at BGI Genomics.

2.8. Elimination of pKD46 Plasmid and Resistance Gene

To eliminate the pKD46 plasmid and its associated ampicillin resistance gene, the gene-deficient strain was
cultured overnight in LB broth containing 25 µg/mL chloramphenicol at 42 °C with shaking (200 rpm). A
20 µL aliquot of the culture was streaked onto LB agar plates containing chloramphenicol, and single
colonies were selected for further propagation. The selected colonies were then streaked onto LB agar
supplemented with both chloramphenicol (25 µg/mL) and ampicillin (50 µg/mL) to screen for strains
capable of growing only on chloramphenicol, indicating successful loss of the temperature-sensitive pKD46
plasmid.

After obtaining the knockout strain devoid of pKD46, competent cells were prepared. The pCP20 plasmid
(carrying ampicillin and chloramphenicol resistance, as well as the FLP recombinase gene) was introduced
via chemical transformation. Transformants were selected on LB agar plates containing both ampicillin (50
µg/mL) and chloramphenicol (25 µg/mL). Following transformation, cells were cultured in LB broth at
30 °C with shaking (180 rpm) for 8 h, after which the temperature was shifted to 42 °C for overnight
incubation with continued shaking to eliminate the pCP20 plasmid. The culture was then streaked onto LB
agar plates, and single colonies were selected. Finally, the expanded culture was streaked onto double-
antibiotic LB agar plates (ampicillin + chloramphenicol) and non-resistant LB agar plates to isolate colonies
that grew exclusively on the non-selective medium, confirming complete plasmid elimination (34).

2.9. Determination of Reducing Sugar Using the 3,5-Dinitrosalicylic Acid Method

E. coli, *E. coli*/pET28a, *E. coli*/pET-sucAC, *E. coli*/Δ*sucC*, and Δ*sucC*/pET-sucAC were pre-cultured to
logarithmic growth phase. Each culture was inoculated at 1% (v/v) into M9 liquid medium and incubated
in a constant-temperature shaker at 30 °C with shaking at 140 rpm. After centrifugation (8,000 × g, 10 min,
4 °C) to remove bacterial cells, the supernatant was collected.

Reducing sugar content in the fermentation broth was determined using the 3,5-dinitrosalicylic acid (DNS) colorimetric method (15). Briefly, 0.5 mL of sample was mixed thoroughly with 1.0 mL of DNS reagent, heated in a boiling water bath for 5 min for color development, and then cooled rapidly to room temperature. The absorbance was measured at 600 nm. Reducing sugar concentration in each sample was calculated based on a glucose standard curve (0–2.0 mg/mL, $R^2 \geq 0.999$). These values reflected the sugar metabolism level and carbon source utilization of different strains under saline-alkali stress conditions. All measurements were performed in triplicate.

2.10. Growth of Different Strains Under Saline-Alkali Stress

E. coli, *E. coli*/pET28a, *E. coli*/pET-sucAC, *E. coli*/Δ*sucC*, and Δ*sucC*/pET-sucAC were pre-cultured to logarithmic growth phase. Each strain was then inoculated at the same initial cell density ($OD_{600} \approx 0.1$) into saline-alkali stress media containing different concentrations of NaCl (0.5, 0.75, 1.0, 1.25, and 1.5 M) and adjusted to various pH values (7.0, 8.0, 8.5, 9.0, and 9.5). Cultures were incubated in a constant-temperature shaker at 30 °C with shaking at 140 rpm. Samples were taken every 8 h to measure absorbance at OD_{600} . Monitoring continued until strains entered the decline phase, and growth curves were plotted. The growth differences and tolerance capacities of the strains under saline-alkali stress were comprehensively compared. All experiments were performed in triplicate.

2.11. Succinyl-CoA Assay

Cells of *E. coli*, *E. coli*/pET28a, *E. coli*/pET-sucAC, *E. coli*/Δ*sucC*, and Δ*sucC*/pET-sucAC were harvested at early stationary phase after growth under saline-alkali stress conditions (1.0 M NaCl, pH 9.0). Intracellular metabolites were extracted following low-temperature centrifugation ($8,000 \times g$, 10 min, 4 °C), methanol quenching (60% v/v methanol, –20 °C, 30 min), and bead-beating disruption (BioSpec Mini-Beadbeater-24, Bartlesville, OK, USA). The intracellular succinyl-CoA content was determined using a commercial enzyme-linked immunoassay kit (Succinyl-CoA ELISA Kit, MyBioSource, San Diego, CA, USA) according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader (BioTek ELx808). Succinyl-CoA levels of the five strains were compared to assess the effects of the *sucA* and *sucC* genes on an intermediate metabolite of the tricarboxylic acid cycle. This metabolic analysis verified the roles of *sucA* and *sucC* in the response to saline-alkali stress. All measurements were performed in triplicate.

2.12. Statistical Analysis

All experiments were performed in triplicate. Data were compiled using Microsoft Excel (Microsoft Corp., Redmond, WA, USA), and statistical analysis and graphing were carried out using Origin 8.0 (OriginLab Corp., Northampton, MA, USA) and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Results are expressed as mean $\pm$ standard deviation (SD). Statistical comparisons among multiple groups were performed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Statistical significance was defined as $p < 0.05$. Significance levels are indicated as: * $p < 0.01$, ** $p < 0.001$, and *** $p < 0.0001$; ns indicates not significant ($p > 0.05$).

3. Results

3.1. Isolation and Identification of Strain P3

The colony morphology of strain P3 was examined on LB agar medium. Colonies appeared smooth, circular, slightly viscous, readily pickable, large, and milky white, with diameters ranging from 0.3 to 0.5 cm (Fig. 2-a). Gram staining revealed short rod-shaped cells that appeared red under light microscopy, leading to the preliminary identification of strain P3 as a Gram-negative bacterium (Fig. 2-b).

Genomic DNA of strain P3 was extracted using the phenol-chloroform method. Using the extracted DNA as template, the 16S rRNA gene was amplified with universal primers 27F and 1492R. Sequencing of the amplified product yielded a 1,536 bp fragment (Fig. 3-a). BLASTn analysis against the NCBI GenBank database revealed that strain P3 shared high sequence similarity with members of the genus *Enterobacter*. A phylogenetic tree was constructed using MEGA 11 software (Fig. 3-b), which demonstrated that strain P3 exhibited 100% 16S rRNA gene sequence identity with *Enterobacter kobei*. Based on morphological, physiological, biochemical, and molecular evidence, strain P3 was definitively identified as *Enterobacter kobei*. The complete genome sequence has been deposited in the National Microbiology Data Center under accession number NMDC60214231.

3.2. Growth Characteristics Under Different Environmental Stresses

3.2.1. Growth of *E. kobei*. P3 strain under a across range of temperatures.

The growth of strain P3 was examined in LB liquid medium across a range of temperatures. Strain P3 grew rapidly at 25 °C, 30 °C, 35 °C, and 40 °C, while slower growth was observed at 10 °C, 15 °C, and 20 °C. These results indicated that strain P3 is capable of growth at low temperatures and was preliminarily characterized as a psychrotolerant bacterium. Growth curves measured at 25 °C, 30 °C, 35 °C, and 40 °C are presented in Fig. 4-a. The strain exhibited optimal growth at 25 °C and 30 °C, with progressively reduced growth rates at 35 °C and 40 °C. The growth trend clearly demonstrated that increasing temperature retarded the growth of strain P3, suggesting that elevated temperatures exert an inhibitory effect. Accordingly, 30 °C was selected as the optimal growth temperature for subsequent experiments.

Principal component analysis of the growth profiles separated the four temperature groups along PC1 (80.4% of variance), whereas PC2 (11.0%) captured additional within-culture differences, together accounting for 91.5% of the total variance Fig. 4-b. The loading structure indicated that cell densities at 16–28 h (mid- to late-exponential phase) contributed most strongly to PC1, i.e., to the temperature-dependent discrimination of growth performance. Collectively, these data demonstrate that *E. kobei* P3 grows most efficiently at 25–30 °C, retains substantial growth at 35 °C, and is comparatively impaired at 40 °C, consistent with a psychrotrophic-to-mesophilic lifestyle relevant to its food-associated and clinical ecology.

3.2.2. Growth of *E. kobei*. P3 strain under a saline-alkali stress.

To evaluate the growth of strain P3 under saline-alkali stress, growth curves were measured across a gradient of NaCl concentrations (0.75, 1.0, 1.25, and 1.5 M) and pH values (8.5, 9.0, 9.5, and 10.0). As illustrated in Fig. 5, strain P3 exhibited robust growth under moderate alkaline conditions (pH 8.5 and 9.0) combined with lower salt concentrations (0.75 M and 1.0 M NaCl), characterized by a short lag phase and high maximum biomass ($OD_{600} > 1.2$). However, growth performance was progressively inhibited as

salinity and alkalinity increased. At a constant pH, elevating the NaCl concentration from 0.75 M to 1.5 M resulted in a significantly prolonged lag phase and a reduced growth rate. Similarly, increasing the pH exerted a compounding inhibitory effect. At pH 9.5, growth was substantially delayed, and no detectable growth was observed at 1.5 M NaCl. Under the most severe condition (pH 10.0), the strain could only sustain growth at 0.75 M and 1.0 M NaCl, with the lag phase extending beyond 30 h and 50 h, respectively. Furthermore, growth was completely arrested at 1.25 M and 1.5 M NaCl when the pH reached 10.0, indicating that this represents the upper limit of its saline-alkali tolerance.

Overall, strain P3 demonstrated a clear dose-dependent sensitivity to both salt and alkaline stress. Based on its ability to thrive at moderate salt concentrations (up to 1.0 M NaCl) while being significantly inhibited at higher salinities (1.5 M NaCl), strain P3 is classified as a moderately halotolerant bacterium according to established microbiological criteria.

3.3 Differential Gene Expression and Functional Enrichment Analysis

3.3.1. Transcriptomic profiling

Transcriptomic profiling revealed progressively broader transcriptional responses in *Enterobacter kobei* P3 with increasing stress intensity. Pairwise comparisons of X2 vs. X1 (Fig. 6-a), X3 vs. X2 (Fig. 6-b), and X3 vs. X1 (Fig. 6-c) identified 384, 789, and 1,448 differentially expressed genes (DEGs) respectively. These comprised 241, 511, and 800 upregulated genes and 143, 278, and 648 downregulated genes (Fig. 6-d). The increasing number of DEGs across comparisons, together with the consistent predominance of upregulated genes, indicates extensive transcriptional reprogramming in response to escalating salt-alkali stress.

3.3.2. GO annotation

GO annotation classified DEGs into biological process (BP), cellular component (CC), and molecular function (MF) categories (Fig. 7-a). Across the three comparisons, 1,143 upregulated and 677 downregulated genes were annotated. Among the 47 genes consistently upregulated across all comparisons, GO enrichment was mainly associated with oxidoreductase activity, pyruvate metabolism, generation of precursor metabolites and energy, glycolysis, and the glycerol-3-phosphate dehydrogenase complex. Catalytic and binding activities predominated in MF, whereas cellular and metabolic processes were the major BP categories.

3.3.3. KEGG analysis

KEGG analysis identified metabolism as the predominant functional category, particularly carbohydrate, amino acid, energy, and cofactor/vitamin metabolism, with additional representation of membrane transport, signal transduction, translation, and protein processing (Fig. 7-b). The 47 common upregulated genes were enriched in glycolysis/gluconeogenesis, the pentose phosphate pathway, pyruvate metabolism, the TCA cycle, carbon fixation in prokaryotes, and methane metabolism. Collectively, these results indicate that increasing salt-alkali stress was associated primarily with transcriptional remodeling of central carbon metabolism, energy metabolism, and redox-related processes, potentially supporting metabolic activity and cellular homeostasis in P3.

3.4. Bioinformatics Analysis of *sucA* and *sucC*

3.4.1. Metabolic pathway map of *sucA* and *sucC*

The genes associated with glycolysis and the TCA cycle were prominently represented under salt-alkali stress. As shown in (Fig. 8) 26 genes in the TCA-cycle pathway were upregulated. Among these, *sucA* and *sucC*, which encode components of the 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase complexes, respectively, were selected for further functional investigation because of their potential involvement in central carbon metabolism and stress adaptation.

Bioinformatics analysis provided significant insights into the genes *sucA* and *sucC*, highlighting their essential roles within the TCA cycle. These genes encode key enzymatic components involved in the conversion of α -ketoglutarate to succinyl-CoA, a critical step in central carbon metabolism. Notably, *sucA* and *sucC* are predicted to function as transcriptional regulators that modulate succinyl-CoA levels in response to saline-alkali stress. Their coordinated expression may facilitate the rerouting of metabolic flux toward the synthesis of compatible solutes or cell wall components, thereby enhancing bacterial tolerance under adverse conditions. These findings suggest that *sucA* and *sucC* are not only involved in energy metabolism but also play regulatory roles in stress adaptation.

3.5. Structural Domain Analysis of SucA and SucC Proteins

Bioinformatics analysis of the target genes was performed using *Escherichia coli* K-12 MG1655 as the reference strain. Fig. S1 shows the genomic organization of the *sucA* and *sucC* genes with their upstream and downstream regions. The *sucA* gene (Gene ID: 945714) is 2,808 bp in length and encodes a protein of 933 amino acids. The deduced SucA protein has a molecular mass of 105.08 kDa, a theoretical isoelectric point (pI) of 6.217, and an instability index of 42.43, indicating that it is an unstable protein (instability index > 40). The *sucC* gene (Gene ID: 945716) is 1,167 bp in length and encodes a protein of 388 amino acids. The SucC protein has a molecular mass of 41.4 kDa, a pI of 5.37, and an instability index of 30.24, indicating a stable protein. Hydrophilicity and hydrophobicity analyses of the SucA and SucC proteins were performed using ProtScale Fig. S2, demonstrating that both proteins are hydrophilic.

Secondary structure prediction using SOPMA revealed that the SucA protein predominantly adopts three secondary structure types: α -helices (383 amino acids, 41.50%), extended strands/ β -sheets (118 amino acids, 12.65%), and random coils (432 amino acids, 46.30%). For the SucC protein, α -helices comprised 177 amino acids (45.62%), extended strands contained 78 amino acids (20.10%), and random coils consisted of 133 amino acids (34.28%). Among the 20 common amino acids, alanine exhibited the highest proportion in both SucA (8.3%) and SucC (12.1%) as shown in Fig. S3.

Domain analysis of the amino acid sequences was performed using InterPro, with predicted functional domains shown in Fig. S4. The SucA protein contains a typical 2-oxoacid dehydrogenase E1 domain and a thiamine pyrophosphate (TPP)-binding domain. These two domains constitute the core functional region of the E1 subunit of the α -ketoglutarate dehydrogenase complex (α -KGDH), which is primarily responsible for catalyzing the decarboxylation of α -ketoglutarate. This step represents the critical structural basis for the conversion of α -ketoglutarate to succinyl-CoA in the TCA cycle. SucA, as a key subunit of the α -KGDH

complex, catalyzes this conversion, thereby maintaining the normal metabolic flux of the TCA cycle. SucC serves as the α subunit of succinyl-CoA synthetase (SCS), which participates in the reversible conversion between succinyl-CoA and succinate while generating ATP via substrate-level phosphorylation. Together, SucA and SucC ensure intracellular energy supply and homeostasis of intermediate metabolites, which may represent an important molecular basis for the strain's response to saline-alkali stress.

Subcellular localization and transmembrane domain analysis were carried out using Soft berry and DeepTMHMM. Prediction results indicated that both SucA and SucC proteins are localized in the cytoplasm, consistent with their catalytic roles as core enzyme subunits of the TCA cycle. This localization supports their central function in intracellular energy metabolism. Under saline-alkali stress, processes such as maintaining cell membrane integrity, sodium ion efflux, and osmotic balance all require substantial energy; thus, the efficiency of the TCA cycle is closely related to the strain's ability to tolerate saline-alkali conditions.

3.6. Cloning and Expression of *sucA* and *sucC*

The amplified DNA fragments were purified and integrated into the pUC18 vector, generating recombinant plasmids designed for expression in a biological system. These recombinant plasmids were introduced into competent *E. coli* cells via chemical transformation, and plasmid DNA was subsequently extracted for further analysis. Additional verification was conducted through plasmid mapping of the engineered expression vector and double restriction enzyme digestion. These analyses confirmed the structural integrity of the recombinant plasmids. The combination of electrophoretic profiles and sequencing results provided conclusive evidence of the successful creation of the recombinant expression strains (Fig. S5, Fig. S6).

The recombinant strains carrying pUC-*sucA* or pUC-*sucC* partially restored salt tolerance of *E. coli* KNabc compared to the empty vector control. However, these strains only supported normal growth below 0.15 M NaCl, with severe growth restriction at higher concentrations. This suggests that expression of either gene alone confers limited improvement and cannot fully compensate for the energy metabolism defect under high salt stress. To test whether co-expression of *sucA* and *sucC* enhances tolerance, a recombinant plasmid pET-*sucAC* was constructed for subsequent functional validation.

3.6.1. Construction and verification of *sucA*- and *sucC*-overexpressing strains

To investigate the functional roles of *sucA* and *sucC*, each gene was cloned into pET28a by homologous recombination and introduced into *E. coli* KNabc, yielding pET-*sucA* and pET-*sucC*. The target genes were amplified and purified as described in Section 2.2.4.5, then ligated into linearized pET28a using a homologous recombination kit. Recombinant plasmids were verified by double digestion with *EcoRV* and *XhoI*, which produced the expected two bands (Fig. S7), and further confirmed by sequencing (BGI Genomics). Verified constructs were preserved and subsequently transferred into *E. coli* BL21 to generate the overexpression strain *E. coli*/pET-*sucAC*. qRT-PCR (Fig. 9) confirmed successful overexpression of both *sucA* and *sucC*.

Relative transcript levels differed significantly among the six groups (one-way ANOVA: $F(5,12) = 51.88$, $p = 1.03 \times 10^{-7}$; values are mean $\pm$ SD, $n = 3$). Tukey's HSD resolved three homogeneous subsets. WT-*sucA* (1.00 ± 0.11) and WT-*sucC* (1.01 ± 0.20) formed the lowest subset (c). The OR-*sucA* clones (2.32 ± 0.26 and 2.05 ± 0.21) formed an intermediate subset (b) and did not differ significantly from each other ($p = 0.81$). The OR-*sucC* clones (3.26 ± 0.40 and 3.67 ± 0.33) formed the highest subset (a), again with no significant clone-to-clone difference ($p = 0.44$). Two-way ANOVA confirmed significant main effects of genotype ($F(1,14) = 151.3$, $p < 0.0001$) and gene ($F(1,14) = 37.6$, $p = 3 \times 10^{-5}$), as well as a significant genotype $\times$ gene interaction ($F(1,14) = 18.4$, $p = 7.5 \times 10^{-4}$), indicating that overexpression increased *sucC* transcript levels more strongly than *sucA* (≈ 3.4 -fold vs ≈ 2.2 -fold over WT). Exploratory PCA (PC1 = 96.4%, PC2 = 3.1%) separated WT from OR samples and ranked groups as WT < OR-*sucA* < OR-*sucC*; independent clones overlapped, supporting clone-to-clone reproducibility. Collectively, these results demonstrate that *sucA* and *sucC* were successfully overexpressed, with *sucC* exhibiting the stronger transcriptional response.

3.7. Physiological Characterization of Recombinant Strains Under Saline-Alkali Stress

Physiological and biochemical characterization of the recombinant expression plasmid pET-*sucAC* was conducted, and the results are presented in Fig. 10. Compared with the empty vector control (KNabc/pET-28a) and the strains expressing either *sucA* or *sucC* alone, co-expression of *sucA* and *sucC* in KNabc/pET-*sucAC* resulted in consistently higher growth under saline-alkali stress.

Under increasing NaCl concentrations (0.05–0.3 M), the co-expression strain maintained robust growth up to 0.3 M NaCl, whereas KNabc/pET-*sucA* and KNabc/pET-*sucC* only grew moderately at NaCl concentrations below 0.15 M and showed marked growth inhibition at 0.2 M and above (Fig. 10-a). Similarly, when challenged with increasing pH from 6.5 to 9.0, the co-expression strain exhibited superior growth at pH 8.5 and pH 9.0, while the single-gene strains and the vector control displayed a sharp decline in growth under the same alkaline conditions (Fig. 10-b). These observations indicate that co-expression of *sucA* and *sucC* synergistically enhances the tolerance of *E. coli* KNabc to both high salinity and high alkalinity. This synergistic effect likely compensates for the energy metabolism defect of the host strain by restoring the complete function of the α -ketoglutarate dehydrogenase complex and succinyl-CoA synthetase in the TCA cycle.

3.8. Construction and Verification of the *sucC* Deletion Mutant

The successful construction of the *E. coli* Δ *sucC* deletion mutant strain is presented in Fig S8. PCR verification was performed using the primers *sucC*-F/*sucC*-R (lanes 2 and 3) and *cm*-F/*cm*-R (lane 4). Lane 1 served as a blank control with ddH₂O as the template for both primer pairs. Lanes 2, 3, and 4 used *E. coli* K-12 as the template, while lanes 5 and 6 used *E. coli* Δ *sucC* as the template. Successful replacement of the *sucC* gene by the targeting fragment was indicated by the following results: the *sucC* gene fragment was detected in lane 3 but not in lane 4; the chloramphenicol resistance gene fragment was absent in lane 5 but present in lane 6. These findings collectively confirm that the resistance cassette was correctly inserted and replaced the *sucC* gene. Furthermore, sequencing results showed 100% identity with the chloramphenicol resistance sequence of the pKD3 plasmid, confirming the successful construction of the *sucC* gene deletion mutant strain of *E. coli*.

3.9. Construction of the Complementary Strain

The expression plasmid pET-*sucAC* was extracted from the overexpression strain *E. coli*/pET-*sucAC* and transformed into the defective strain *E. coli* Δ *sucC* by chemical transformation to generate the gene complementary strain Δ *sucC*/pET-*sucAC*. Positive clones were selected on LB medium containing 50 μ g/mL kanamycin. Plasmids were extracted and verified by agarose gel electrophoresis. Correct plasmids, as indicated by the expected band sizes, were further confirmed by double restriction enzyme digestion with *EcoRV* and *XhoI* (Fig. S9). The successfully complemented strain was preserved for subsequent analyses.

3.10. Reducing Sugar Content Analysis of Recombinant Strains

To further verify the enhancing effect of *sucA* and *sucC* co-expression on saline-alkali tolerance, residual glucose concentrations in culture supernatants were determined using the 3,5-dinitrosalicylic acid (DNS) method (Fig. 11). Five strains were examined: wild-type *E. coli*, the deletion mutant *E. coli* Δ *sucC*, the complementary strain Δ *sucC*/pET-*sucAC*, the empty vector control *E. coli*/pET-28a, and the overexpression strain *E. coli*/pET-*sucAC*.

After 12 h and 24 h of growth in M9 glucose medium, the residual glucose concentration of the deletion mutant was significantly higher than that of the other strains at both time points, whereas the overexpression strain consistently showed the lowest residual glucose. At 24 h, the residual glucose levels of the overexpression strain and the empty vector control were comparable, and the complementary strain exhibited a marked reduction compared with the deletion mutant, reaching levels close to those of the empty vector control. These results indicate that co-expression of *sucA* and *sucC* enhances saline-alkali tolerance by improving glucose utilization efficiency, and the complementation experiment further supports the essential role of these genes in maintaining central carbon metabolic homeostasis under stress conditions.

3.11. Growth Performance of Recombinant Strains Under Salt Stress

To assess the effects of *sucA* and *sucC* on saline-alkali tolerance, growth curves of the five strains were measured under increasing NaCl concentrations (0, 0.25, 0.5, 0.75, and 1.0 M) (Fig. 12). At each salt concentration, growth of all strains initially increased rapidly and then reached a plateau, with overall growth rate and final biomass decreasing as salinity increased. Under the same NaCl concentration, the overexpression strain *E. coli*/pET-*sucAC* exhibited significantly higher growth rate and final OD₆₀₀ than the wild-type and empty vector strains, indicating enhanced saline-alkali tolerance. In contrast, the deletion mutant *E. coli* Δ *sucC* showed the slowest growth and lowest biomass, whereas the complementary strain Δ *sucC*/pET-*sucAC* restored growth to levels comparable to those of the wild-type and empty vector strains, significantly outperforming the deletion mutant.

Similarly, the effect of alkaline stress was examined at pH values ranging from 8.0 to 9.5 (Fig. 13). Growth of all strains followed a similar pattern of rapid increase followed by a stationary phase, with growth rate and final biomass declining as pH increased, confirming that alkaline stress also significantly inhibited growth. Compared with salt stress, the differences among strains under alkaline stress were relatively

smaller, but the overall trend remained consistent. These results demonstrate that co-expression of *sucA* and *sucC* significantly enhances tolerance to both salt and alkaline stress, whereas deletion of *sucC* impairs stress resistance, confirming the important regulatory roles of *sucA* and *sucC* in the response to saline-alkali stress.

3.12. Intracellular Succinyl-CoA Levels in Recombinant Strains

Fig. 14 shows the intracellular succinyl-CoA (SCoA) levels measured by enzyme-linked immunosorbent assay under 0, 0.25, and 0.5 M NaCl to investigate the effect of *sucA* and *sucC* on SCoA levels under saline-alkali stress. As NaCl concentration increased from 0 to 0.5 M, SCoA levels in all strains increased significantly ($p < 0.01$ or $p < 0.001$). The wild-type and empty vector strains showed similar trends with no significant difference, indicating that the empty vector did not affect SCoA metabolism.

The overexpression strain *E. coli*/pET-sucAC exhibited consistently lower SCoA levels than the wild-type and empty vector strains at all salt concentrations, suggesting that co-expression of *sucA* and *sucC* accelerates SCoA catabolism and reduces its accumulation, thereby promoting succinate production for the TCA cycle. In contrast, the deletion mutant *E. coli* Δ *sucC* showed significantly higher SCoA levels than the other strains under each condition, indicating impaired SCoA metabolism, with even greater accumulation under salt stress. The complementary strain Δ *sucC*/pET-sucAC restored SCoA levels to values close to those of the wild-type and empty vector strains, significantly lower than those of the deletion mutant. These results confirm that co-expression of *sucA* and *sucC* effectively restores SCoA metabolic homeostasis and significantly enhances bacterial growth under saline-alkali stress.

4. Discussion

The global expansion of saline-alkaline soils demands bioremediation strategies rooted in the metabolic versatility of extremophilic microorganisms (29, 4). *Enterobacter kobei* P3, isolated from Egyptian saline-alkali soil, grows at 3% NaCl and pH 8.5 and tolerates up to 1.25 M NaCl and pH 9.5 conditions that mirror degraded agricultural lands in arid climates (7). This phenotype makes P3 a biologically relevant model for dissecting salt-alkaline adaptation. Our integrated transcriptomic and functional genetic approach converges on a central finding: the TCA cycle genes *sucA* and *sucC* act as synergistic metabolic regulators that enhance tolerance by accelerating energy production and carbon source utilization.

The transcriptomic analysis revealed progressive metabolic reprogramming of *Enterobacter kobei* P3 under increasing salt-alkali stress, with DEGs increasing from 384 in X2 vs. X1 to 1,448 in X3 vs. X1. The consistently upregulated genes were mainly associated with glycolysis, pyruvate metabolism, oxidoreductase activity, energy generation, and the TCA cycle, indicating that central carbon and energy metabolism are important components of the stress response. Similar metabolic remodeling has been reported in *Enterobacter* under pH stress and in other bacteria exposed to osmotic stress (16, 27).

The TCA cycle was particularly prominent, with 26 associated genes upregulated under salt-alkali stress. Among these, *sucA* and *sucC* encode the E1 component of the 2-oxoglutarate dehydrogenase complex and the ATP-forming component of succinyl-CoA synthetase, respectively. Because the TCA cycle contributes to energy production, precursor supply, and redox balance, their induction suggests that these metabolic

551 steps may contribute to P3 adaptation to salt–alkali stress (23). However, transcript abundance alone does
552 not establish increased metabolic flux or causality.

553 Recent work demonstrates that osmotic stress can remodel central metabolism in a stressor- and organism-
554 dependent manner, while functional analysis identified *sucC* as a potential contributor to osmo-tolerance
555 in *C. glutamicum* (27). Consistent with the transcriptomic response, the elevated succinyl-CoA level
556 observed in stressed wild-type cells provides additional metabolite-level support for altered TCA
557 metabolism. Succinyl-CoA can be converted to succinate by succinyl-CoA synthetase, after which succinate
558 enters the respiratory branch through succinate dehydrogenase. Thus, the coordinated upregulation of
559 *sucA* and *sucC* together with altered succinyl-CoA levels supports their potential involvement in metabolic
560 adaptation, while the subsequent genetic characterization is required to establish their specific
561 contributions to salt–alkali tolerance.

562 The functional complementation and knockout experiments offer critical genetic proof that this
563 transcriptional response is adaptive rather than incidental. The modest gain conferred by single-gene
564 expression suggests that *sucA* and *sucC* operate within a tightly coupled metabolic sequence: SucA
565 produces succinyl-CoA, which SucC immediately channels toward succinate and GTP generation (26).
566 Disrupting either step creates a bottleneck, whereas co-expression ensures stoichiometric balance. This
567 interpretation is reinforced by growth data, where the *sucAC* overexpression strain sustained exponential
568 growth under 0.5 M NaCl and pH 9.0 conditions that arrested the wild type (13).

569 The Δ *sucC* mutant, by contrast, displayed a sugar accumulation phenotype, leaving abundant glucose
570 unmetabolized (11). This metabolic paralysis likely stems from feedback inhibition: when the TCA cycle is
571 blocked at the succinyl-CoA synthetase step, NADH and ATP depletion signals may suppress glycolytic
572 activity, creating a cycle of energy starvation (17).

573 The structural features of SucA and SucC are consistent with their proposed catalytic roles. SucA harbors
574 a 2-oxoacid dehydrogenase E1 domain and a thiamine pyrophosphate binding domain, characteristic of
575 the E1 subunit of the α -ketoglutarate dehydrogenase complex, which catalyzes the rate-limiting
576 decarboxylation of α -ketoglutarate to succinyl-CoA (26). SucC possesses a CoA-synthetase core domain
577 and an ATP-binding domain, typical of the α subunit of succinyl-CoA synthetase, which catalyzes the
578 reversible conversion of succinyl-CoA to succinate with concomitant ATP or GTP production (28).

579 The cytoplasmic localization of both proteins matches the compartment where the TCA cycle operates. The
580 predominance of α -helices (41.50% in SucA, 45.62% in SucC) provides structural stability for functional
581 domains, while random coils confer conformational flexibility a combination that facilitates efficient
582 substrate binding and catalysis (2).

583 The mechanism by which enhanced TCA cycle flux promotes saline–alkaline tolerance can be framed
584 around three interconnected demands. First, ATP is required for ion extrusion. Na^+/H^+ antiporters, such as
585 the NhaA-type transporters widespread in halotolerant bacteria, depend on the proton motive force and,
586 in some cases, directly on ATP to maintain cytoplasmic Na^+ homeostasis (32). Second, reducing equivalents
587 (NADH, FADH_2) sustain the respiratory chain and help maintain membrane integrity through fatty acid

remodeling (39). Third, TCA intermediates serve as precursors for compatible solutes trehalose, proline, and glycine betaine that counteract osmotic stress (31).

In halophiles such as *Chromohalobacter salexigens*, the biosynthesis of ectoines quantitatively draws off TCA cycle intermediates, and anaplerotic activity is elevated to replenish the cycle (7). A similar coupling may operate in P3: enhanced flux through SucA and SucC could support both energy generation and the biosynthetic demands of osmo-protectant production.

An additional, though speculative, benefit of accelerated TCA cycle activity is pH buffering. The decarboxylation of α -ketoglutarate by the α -KGDH complex consumes protons, and sustained operation of the cycle helps maintain the proton motive force under alkaline conditions (28). This model is consistent with reports that energy metabolism genes play pivotal roles in microbial tolerance to high salinity and alkalinity (35). The direction of TCA cycle regulation under salt stress appears species-specific: in some organisms, salt shock downregulates *sucA* and *sucC*, whereas in others, these genes are induced (37). This contrast underscores the value of functional validation in P3 transcriptomic enrichment alone would not have established causality.

Several limitations merit consideration. The ELISA measurements of succinyl-CoA provide a steady-state snapshot but do not quantify flux; isotope tracing or enzyme activity assays would strengthen the claim that *sucA/sucC* co-expression accelerates TCA cycle turnover (21). The growth and glucose consumption data support a model in which improved energy metabolism underlies enhanced tolerance, but direct measurements of ATP pools, Na^+/H^+ antiporter activity, or compatible solute accumulation would provide a more complete mechanistic picture (32). Additionally, the experiments were conducted in *E. coli* as a heterologous host; whether the same regulatory logic operates in *E. kobei* P3 itself remains to be tested through targeted knockout or knockdown in the native strain (36).

In summary, this study identifies *sucA* and *sucC* as key determinants of saline–alkaline tolerance in *E. kobei* P3. Their co-expression improves glucose utilization, accelerates succinyl-CoA catabolism, enhances ATP production, and thereby supports the energy-dependent mechanisms required for survival under extreme conditions. These findings deepen the molecular understanding of microbial stress adaptation and provide genetic targets for engineering robust strains for agricultural and industrial applications from biofertilizers for saline–alkali soils to microbial cell factories with improved stress tolerance.

5. Conclusion

This study establishes *sucA* and *sucC* as key genetic determinants of saline–alkaline tolerance in *Enterobacter kobei* P3. Transcriptomic profiling linked saline–alkaline stress to enhanced central carbon metabolism, and functional validation confirmed that co-expression of these TCA cycle genes improves glucose utilization, accelerates succinyl-CoA turnover, and elevates ATP production. These metabolic adjustments sustain the energy-dependent processes required for ion homeostasis, compatible solute synthesis, and membrane integrity under extreme conditions.

The growth advantage conferred by *sucA/sucC* co-expression, together with the metabolic paralysis of the Δ *sucC* mutant, provides genetic evidence that these genes are not merely stress-responsive but functionally adaptive. While the heterologous *E. coli* system and steady-state metabolite measurements represent

limitations, the findings offer a mechanistic framework for engineering halotolerant and alkali-tolerant strains. Future work should validate these targets in the native P3 background and quantify metabolic flux to fully resolve the coupling between TCA cycle activity and stress protection. Ultimately, this work expands the genetic toolbox for developing robust microbial inoculants for saline–alkali soils and stress-tolerant cell factories for industrial biotechnology.

CRedit authorship contribution statement

Conceptualization: Yanhong Wang, Jiahui Chen, Beibei Xu, Amro Abdelazez, Heba Abdelmotaal. **Methodology:** Jiahui Chen, Beibei Xu, Yitong Feng, Yaru Zhu, Wenzhao Wang, Ming Yang. **Formal analysis:** Amro Abdelazez, Heba Abdelmotaal, Yitong Feng, Yaru Zhu, Jingxuan Pan, Ming Yang. **Investigation:** Heba Abdelmotaal, Yitong Feng. **Data curation:** Heba Abdelmotaal. **Visualization:** Amro Abdelazez, Heba Abdelmotaal, Wenzhao Wang, Jingxuan Pan. **Supervision:** Yanhong Wang. **Project administration:** Yanhong Wang, Amro Abdelazez. **Funding acquisition:** Yanhong Wang, Jiahui Chen, Beibei Xu. **Writing – original draft:** Amro Abdelazez, Heba Abdelmotaal. **Writing – review & editing:** Amro Abdelazez, Heba Abdelmotaal, Yanhong Wang, Jiahui Chen, Beibei Xu, Yaru Zhu, Wenzhao Wang, Jingxuan Pan, Ming Yang. **Writing – original draft:** Wang Hongyu

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Reference

1. Abdel-motaal, H., Abdelazez, A., Abozaed, S., Wang, Y., El-Aziz, M. A. A., El-Khayat, R. M. and Ahmed, K. R. (2026) Isolation and Characterization of Halotolerant Plant Growth-Promoting Rhizobacteria for Sustainable Agriculture in Saline Soils. *Applied Biochemistry and Biotechnology*.
2. Bothe, S. N. and Zdanowicz, R. (2026) Structural diversity of pyruvate dehydrogenase complexes. *FEBS Letters*, **600**, 1475-1490.
3. Cui, Q., Xia, J., Yang, H., Liu, J. and Shao, P. (2021) Biochar and effective microorganisms promote *Sesbania cannabina* growth and soil quality in the coastal saline-alkali soil of the Yellow River Delta, China. *Science of The Total Environment*, **756**, 143801.
4. Cui, Y., Ning, Z., Li, M., Qin, X., Yue, X., Chen, X., Zhu, C., Sun, H. and Huang, Y. (2025) Microbial network-driven remediation of saline-alkali soils by salt-tolerant plants. *Frontiers in Microbiology*, **Volume 16 - 2025**.

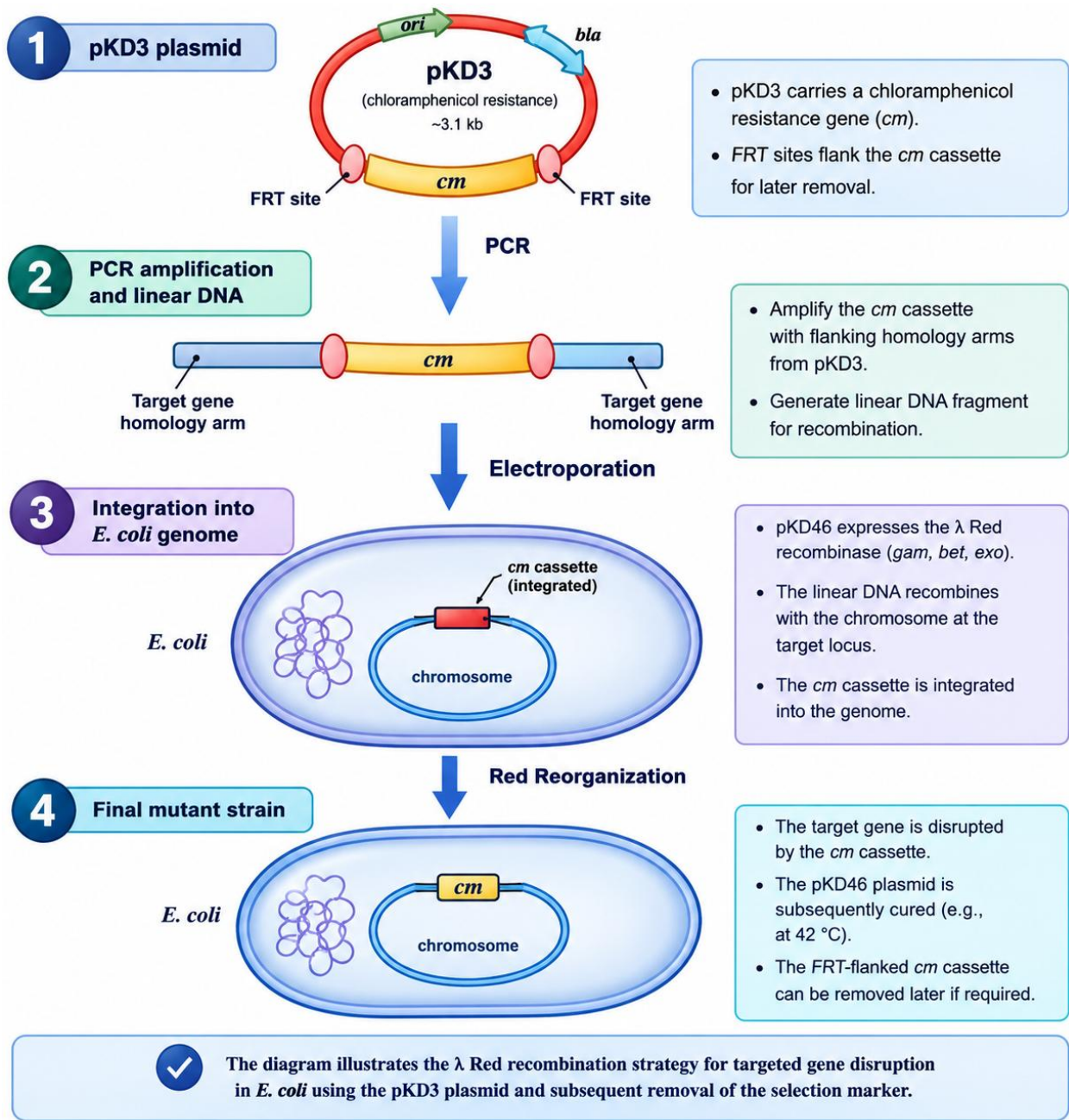
5. Ezenwanne, B. C., Okoye, C. O., Jiang, H., Gao, L., Chen, X., Wu, Y. and Jiang, J. (2026) Microbial strategies for soda saline-alkali soil remediation: The role of haloalkaliphilic bacteria. *Microbiological Research*, **304**, 128410.
6. FAO. (2021) The State of the World's Land and Water Resources for Food and Agriculture—Systems at Breaking Point. Synthesis Report 2023. Food and Agriculture Organization of the United Nations, Rome.
7. Forni, C., Duca, D. and Glick, B. R. (2017) Mechanisms of plant response to salt and drought stress and their alteration by rhizobacteria. *Plant and Soil*, **410**, 335-356.
8. Goszcz, A., Furtak, K., Stasiuk, R., Wójtowicz, J., Musiałowski, M., Schiavon, M. and Dębiec-Andrzejewska, K. (2025) Bacterial osmoprotectants-a way to survive in saline conditions and potential crop allies. *FEMS Microbiol Rev*, **49**.
9. Gunde-Cimerman, N., Plemenitaš, A. and Oren, A. (2018) Strategies of adaptation of microorganisms of the three domains of life to high salt concentrations. *FEMS Microbiol Rev*, **42**, 353-375.
10. Guo, X., Peng, W., Xu, X., Xie, K. and Yang, X. (2023) The Potential of Endophytes in Improving Salt–Alkali Tolerance and Salinity Resistance in Plants. *International Journal of Molecular Sciences*, **24**, 16917.
11. Gusakov, A. V., Kondratyeva, E. G. and Sinitsyn, A. P. (2011) Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities. *Int J Anal Chem*, **2011**, 283658.
12. Ijaz, S. and Hassan, S. (2026), in *Molecular Biology Methods*, (Ijaz, S., Haq, I. U. and Mohamed Ali, H., eds.), Springer Nature Singapore, Singapore, pp. 1-57.
13. Jamali, Z. H., Younas, M. U., Qasim, M., Ali, S., Iqbal, R., Aghayeva, S. and Konvalina, P. (2026) Multi-omics and precision breeding strategies for enhancing biotic and abiotic stress resilience in rice. *Cereal Research Communications*, **54**, 1995-2012.
14. Janda, J. M. and Abbott Sharon, L. (2021) The Changing Face of the Family Enterobacteriaceae (Order: “Enterobacterales”): New Members, Taxonomic Issues, Geographic Expansion, and New Diseases and Disease Syndromes. *Clinical Microbiology Reviews*, **34**, 10.1128/cmr.00174-00120.
15. King, B. C., Donnelly, M. K., Bergstrom, G. C., Walker, L. P. and Gibson, D. M. (2009) An optimized microplate assay system for quantitative evaluation of plant cell wall–degrading enzyme activity of fungal culture extracts. *Biotechnology and Bioengineering*, **102**, 1033-1044.
16. Kumari, K., Sharma, P. K. and Singh, R. P. (2024) The transcriptome response of *Enterobacter* sp. S-33 is modulated by low pH-stress. *Genes Genomics*, **46**, 671-687.
17. Lancaster, M. S. and Graham, B. H. (2023) Succinyl-CoA Synthetase Dysfunction as a Mechanism of Mitochondrial Encephalomyopathy: More than Just an Oxidative Energy Deficit. *International Journal of Molecular Sciences*, **24**, 10725.
18. Li, L., Yang, S., Hu, X., Li, Z. and Chen, H. (2024) The combined application of salt-alkali tolerant phosphate solubilizing microorganisms and phosphogypsum is an excellent measure for the future improvement of saline-alkali soils. *Frontiers in Microbiology*, **Volume 15 - 2024**.
19. Li, Y., Liu, T., Li, F., Li, M., Shen, Z., Zhao, X., Liu, H., Zhang, S. and Cai, J. (2026) Algal–bacterial synergy for saline-alkaline soil bioremediation: mechanisms, advances and challenges. *Environmental Geochemistry and Health*, **48**, 351.
20. Liu, X., Lin, S., Li, C., Ren, S., Tang, Y., Tang, L. and Zhang, Z. (2026) Functional characteristics and distribution of microorganisms in saline and alkaline soils and their probiotic effects: a review. *Microbial Cell Factories*, **25**, 39.
21. Ly, C. H., Lynch, G. S. and Ryall, J. G. (2020) A Metabolic Roadmap for Somatic Stem Cell Fate. *Cell Metabolism*, **31**, 1052-1067.

- 710 22. Ma, X., Chen, B., Liang, J., Shao, L., Xu, X., Qiu, H., Cao, X. and Zhao, L. (2026) Beyond universal
711 application: dissecting the differential amelioration and carbon sequestration performance of
712 biochar in chloride-, sulfate-, and soda saline-alkali soils. *Geoderma*, **469**, 117809.
- 713 23. MacLean, A., Legendre, F. and Appanna, V. D. (2023) The tricarboxylic acid (TCA) cycle: a
714 malleable metabolic network to counter cellular stress. *Crit Rev Biochem Mol Biol*, **58**, 81-97.
- 715 24. Meng, Q., Zhang, C., Zhao, J., Zhang, J., Lu, Y. and Liu, F. (2026) Saline-alkali soil amelioration in
716 China: A systematic review and evidence synthesis. *Agricultural Water Management*, **327**,
717 110296.
- 718 25. Mishra, P., Mishra, J. and Arora, N. K. (2021) Plant growth promoting bacteria for combating
719 salinity stress in plants - Recent developments and prospects: A review. *Microbiol Res*, **252**,
720 126861.
- 721 26. Nemeria, N. S., Zhang, X., Leandro, J., Zhou, J., Yang, L., Houten, S. M. and Jordan, F. (2021)
722 Toward an Understanding of the Structural and Mechanistic Aspects of Protein-Protein
723 Interactions in 2-Oxoacid Dehydrogenase Complexes. *Life*, **11**, 407.
- 724 27. Ni, X., Gao, N., Hu, W., Guo, X., Liu, J., Chen, J., Cai, N., Zhao, X., Sun, J., Li, Y., Zhu, Y. and Zheng,
725 P. (2026) Deciphering mechanisms of stress-induced metabolic shifts in *Corynebacterium*
726 *glutamicum* by transcriptomics and contextualised modelling. *Bioresour Technol*, **440**, 133406.
- 727 28. Pacesa, M., Pelea, O. and Jinek, M. (2024) Past, present, and future of CRISPR genome editing
728 technologies. *Cell*, **187**, 1076-1100.
- 729 29. Paul, D. and Lade, H. (2014) Plant-growth-promoting rhizobacteria to improve crop growth in
730 saline soils: a review. *Agronomy for Sustainable Development*, **34**, 737-752.
- 731 30. Poolman, B. (2023) Physicochemical homeostasis in bacteria. *FEMS Microbiology Reviews*, **47**,
732 fuad033.
- 733 31. Prasad, S. K. and Singh, R. P. (2026) In-depth genomic investigation of halophilic bacterium
734 *Priestia megaterium* CL-1 isolated from Chilika Salt Lake. *Discover Applied Sciences*.
- 735 32. Vasan, S. K., Noordam, R., Gowri, M. S., Neville, M. J., Karpe, F. and Christodoulides, C. (2019)
736 The proposed systemic thermogenic metabolites succinate and 12,13-diHOME are inversely
737 associated with adiposity and related metabolic traits: evidence from a large human cross-
738 sectional study. *Diabetologia*, **62**, 2079-2087.
- 739 33. Wang, X., Miao, Y., Shen, Y., Lyu, J., Cui, H., Hou, S., Yan, B. and Zhu, H. (2025) Adaptation and
740 assembly of microbial communities under saline-alkaline stress in paddy ecosystems:
741 Implications for nitrogen and carbon cycling. *Environmental Technology & Innovation*, **39**,
742 104329.
- 743 34. Wang, Y., Xu, B., Abdelazez, A., Abdel-motaal, H., Liu, Q., Han, L., Yang, M., Wang, W., Abd El-
744 Aziz, M. A., Alshehry, G., Algarni, E. and Aljumayi, H. (2026) Characterization of the YCjN ABC
745 Transporter in *Escherichia coli*: Role in Maltose and Ethidium Bromide Transport. *Molecular*
746 *Biotechnology*, **68**, 1940-1964.
- 747 35. Yang, W., Zhao, P., Liu, Y., Cao, P., Ji, X., Gao, Y., Li, P. and Cheng, J. (2021) Transcriptome
748 analysis of lncRNA expression patterns in human congenital lung malformations. *BMC*
749 *Genomics*, **22**, 861.
- 750 36. Yu, B. J., Sung, B. H., Lee, J. Y., Son, S. H., Kim, M. S. and Kim, S. C. (2006) *sucAB* and *sucCD* are
751 mutually essential genes in *Escherichia coli*. *FEMS Microbiology Letters*, **254**, 245-250.
- 752 37. Yu, X., Xie, Y., Zhang, X., Ma, C., Liu, L., Zhen, W., Xu, L., Zhang, J., Liang, Y., Zhao, L., Gao, X., Yu,
753 P., Luo, J., Jiang, L.-H., Nie, Y., Yang, F., Guo, J. and Yang, W. (2021) Structural and functional
754 basis of the selectivity filter as a gate in human TRPM2 channel. *Cell Reports*, **37**.
- 755 38. Zhang, S., Li, K., Wang, C., Yang, S., Liu, X. and Pan, K. (2026) Advances in Biological and Physical
756 Salt-Reduction Technologies for Reclaiming Saline-Alkali Land: A Comprehensive Review with an
757 Emphasis on China. *Agronomy*, **16**, 1645.

39. Zhou, H., Shi, H., Yang, Y., Feng, X., Chen, X., Xiao, F., Lin, H. and Guo, Y. (2024) Insights into plant salt stress signaling and tolerance. *Journal of Genetics and Genomics*, **51**, 16-34.

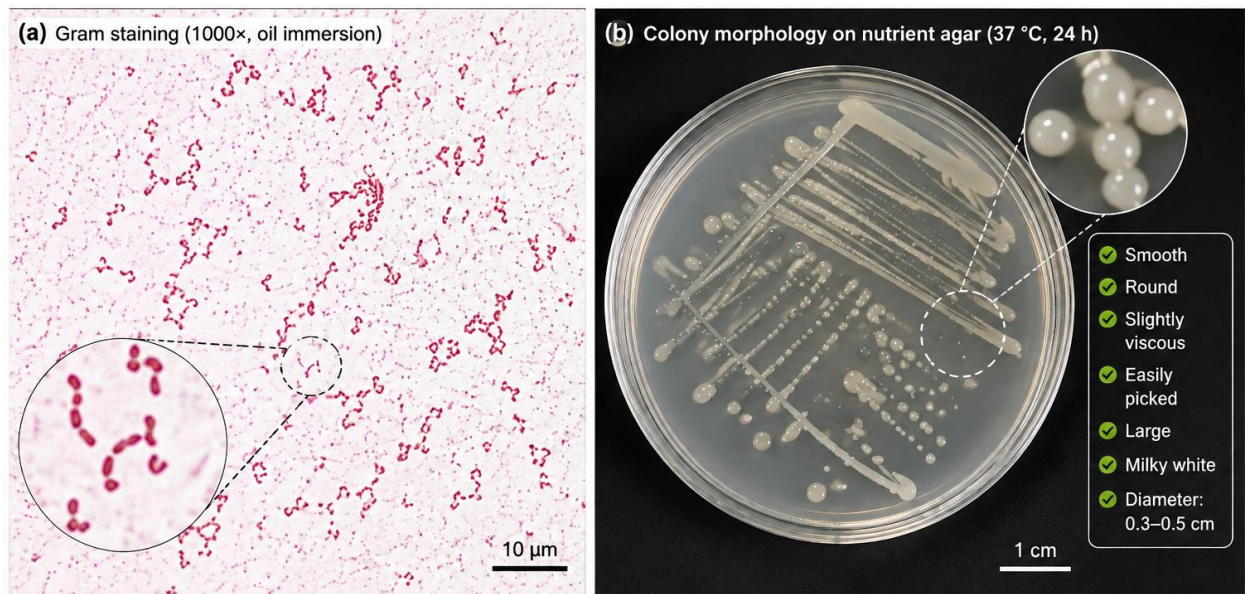
Figure list

Figure 1. Schematic illustration of the pKD3-mediated homologous recombination strategy for targeted gene knockout in *E. coli*.



The linear DNA fragment containing the kanamycin resistance cassette (*kan*) and homologous arms is amplified from the pKD3 plasmid. Following electroporation and Lambda Red recombinase-mediated homologous recombination, the target gene is replaced by the resistance cassette, resulting in a precise gene disruption.

Figure 2. Morphological characterization of *Enterobacter kobei* P3.



a) Gram staining under oil- immersion microscopy shows short rod-shaped cells stained pink- red. Indicating a gram-negative cell wall structure. **b)** Colony morphology on nutrient agar after 24 h at 37 °C

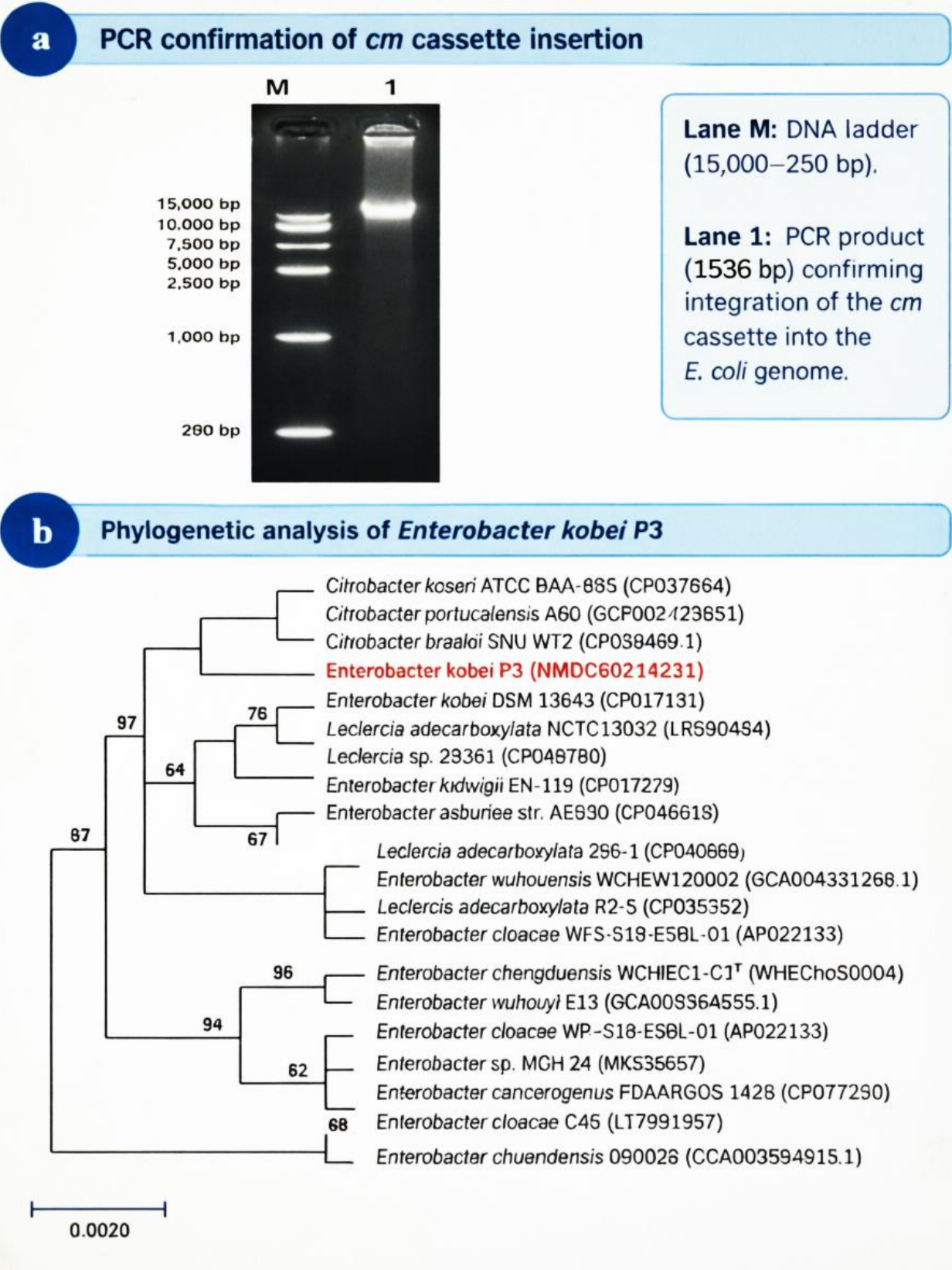
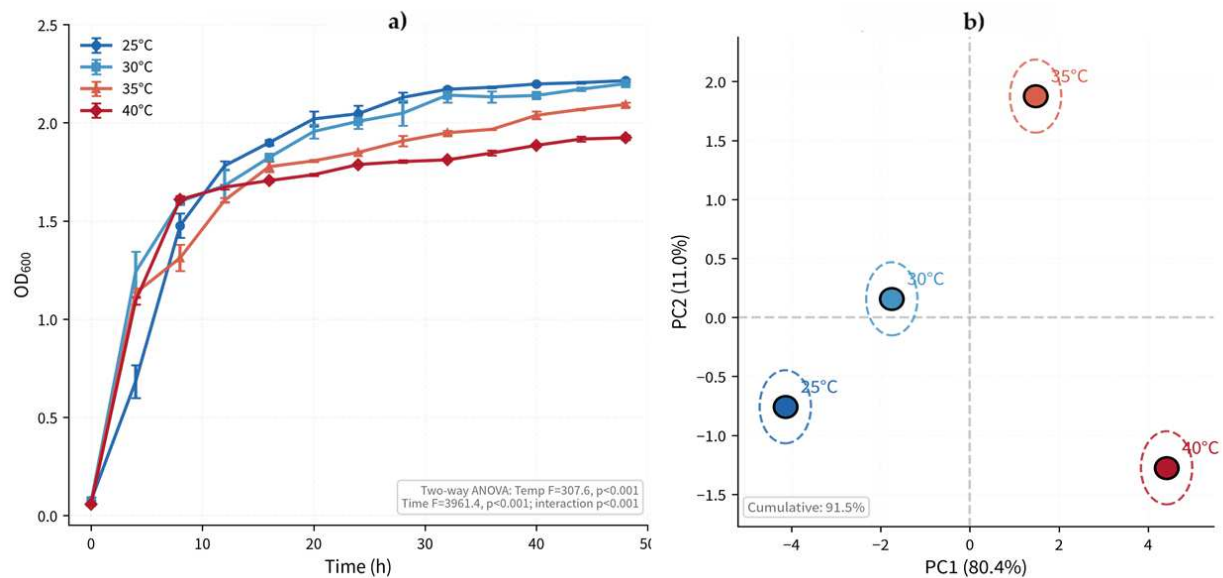


Figure 4. Growth curves of *Enterobacter kobei* P3 under different temperatures



a) Growth curves at 25, 30, 35, and 40°C in LB medium.; b) Principal component analysis of the growth profiles; PC1 and PC2 account for 80.4% and 11.0% of the total variance, respectively (cumulative 91.5%).

Figure 5. Combined effects of NaCl concentration and pH on the growth of *Enterobacter kobei* P3 as different saline-alkaline concentrations

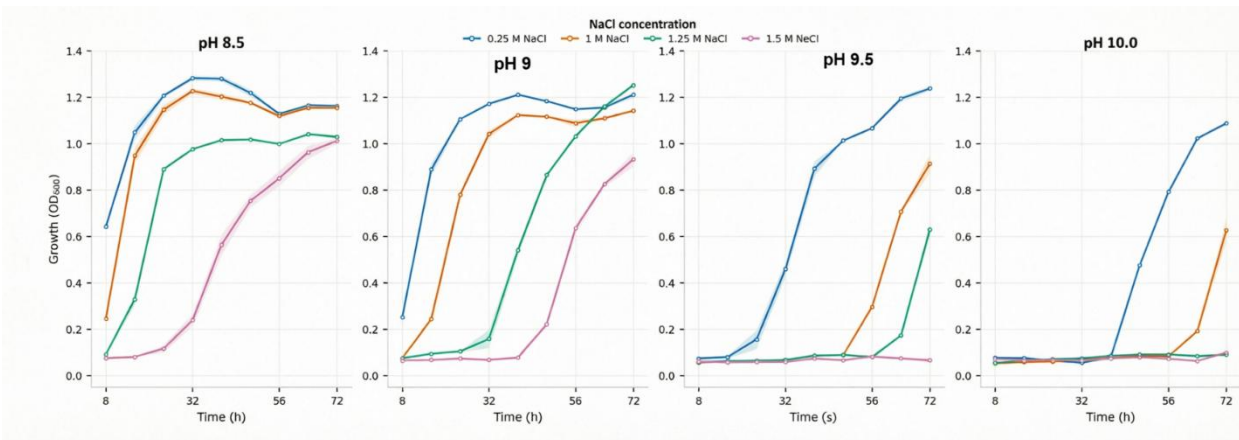
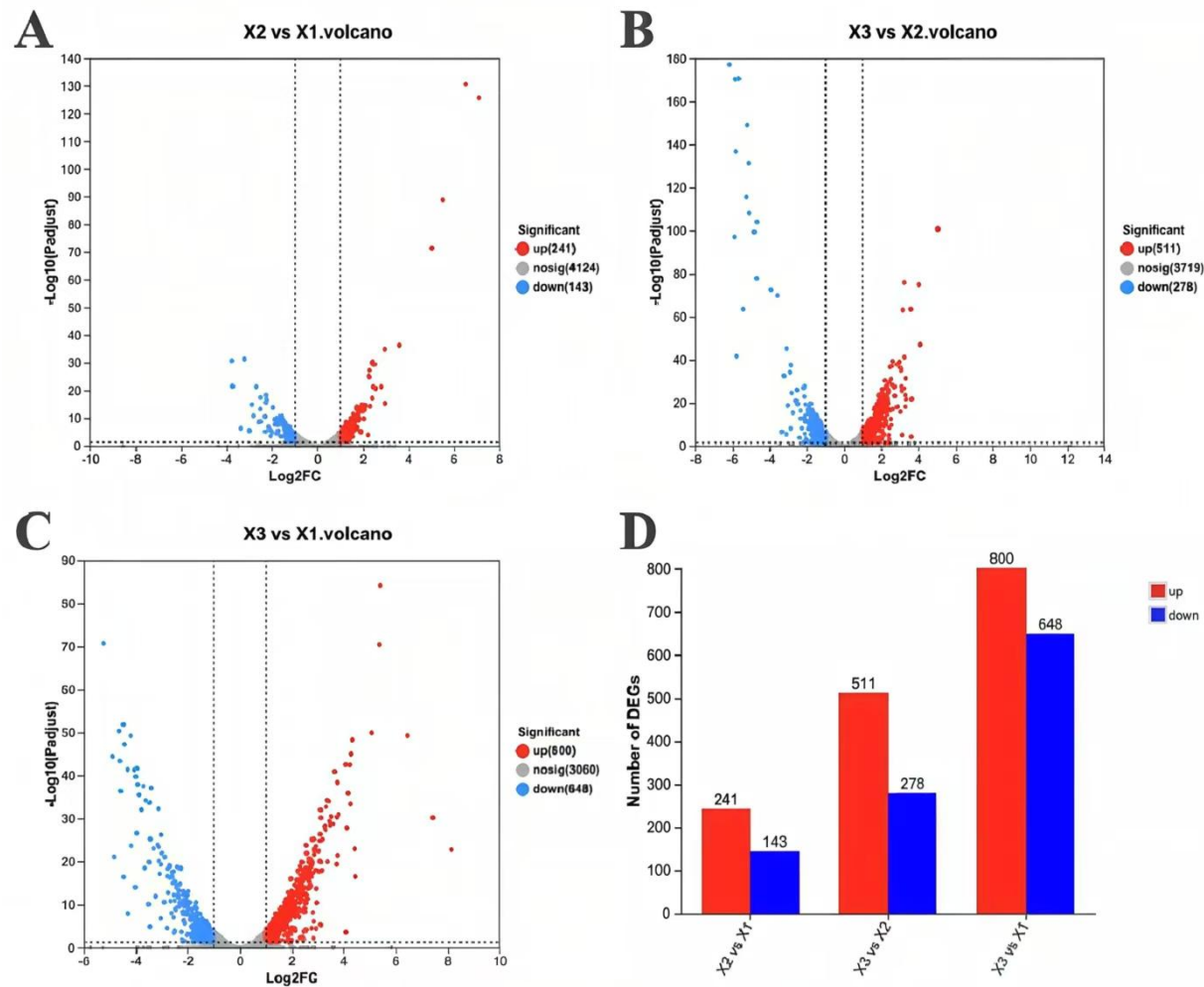
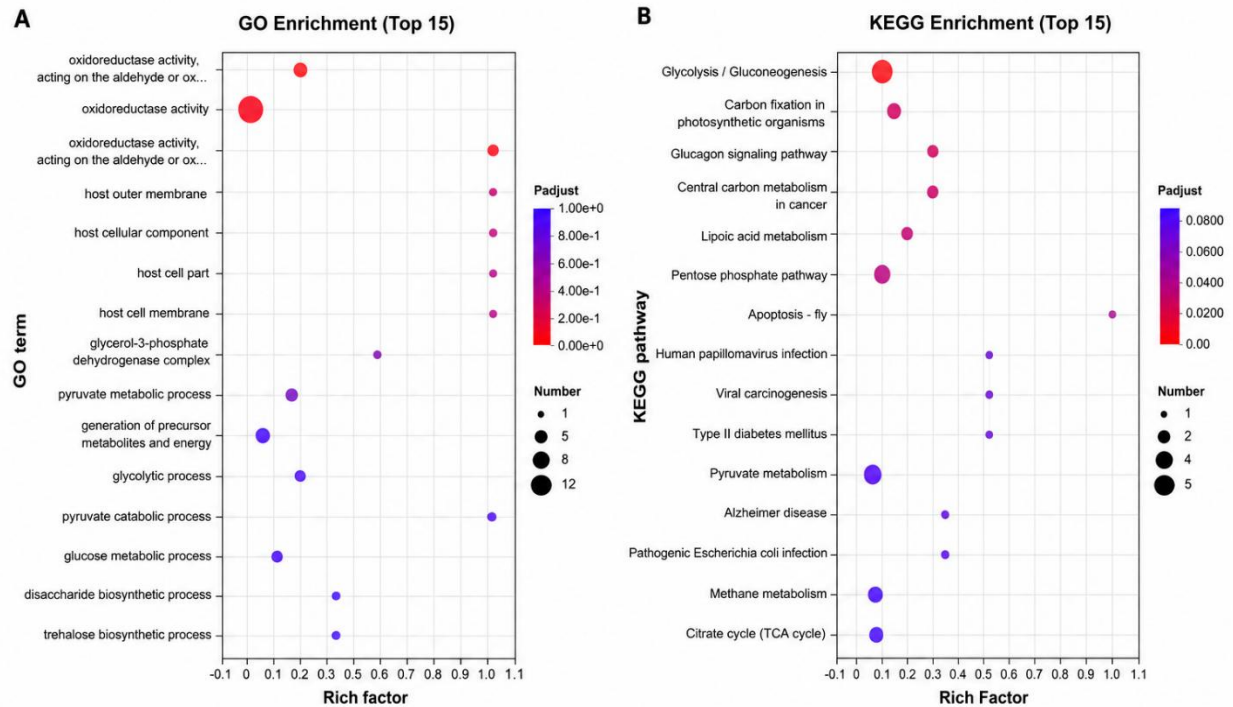


Figure 6. Differential gene expression of *Enterobacter kobei* P3 under increasing salt-alkali stress



(A–C) Volcano plots showing differentially expressed genes (DEGs) in (A) X2 vs. X1, (B) X3 vs. X2, and (C) X3 vs. X1. Red and blue points indicate upregulated and downregulated genes, respectively, while gray points represent genes that were not significantly differentially expressed. Dashed lines indicate the fold-change and significance thresholds. (D) Numbers of upregulated and downregulated DEGs in each comparison. DEGs were defined as $|\log_2 \text{fold change}| \geq 1$ and adjusted $P < 0.05$. X1, low stress (natural pH, 0.25 M NaCl); X2, moderate stress (pH 8.5, 0.75 M NaCl); X3, high stress (pH 9.0, 1.25 M NaCl).

Figure 7. GO and KEGG enrichment analysis of commonly upregulated genes across salt-alkali stress comparisons



(A) Top 15 enriched Gene Ontology (GO) terms. (B) Top 15 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. In both panels, the x-axis represents the Rich Factor (the ratio of the number of DEGs to the total number of background genes annotated in the specific term or pathway). The size of the dots corresponds to the number of enriched genes, and the color gradient indicates the adjusted *P*-value (*Padjust*), with red denoting higher statistical significance (lower *P*-value) and blue denoting lower significance.

Figure 8. Metabolic pathway map of *sucA* and *sucC*

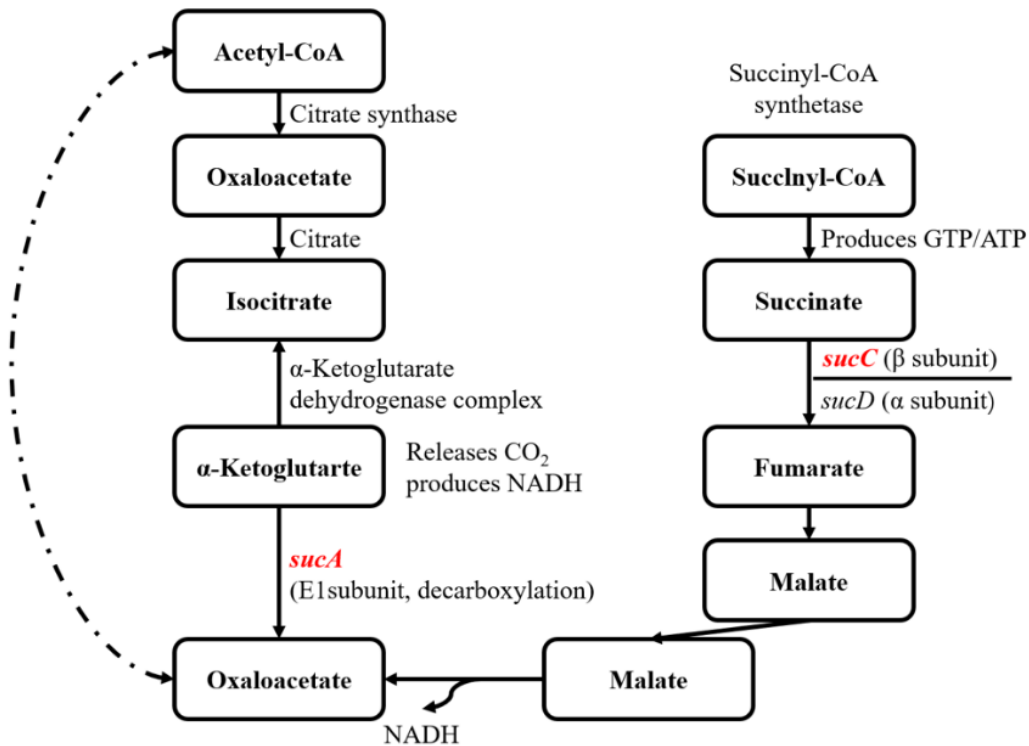
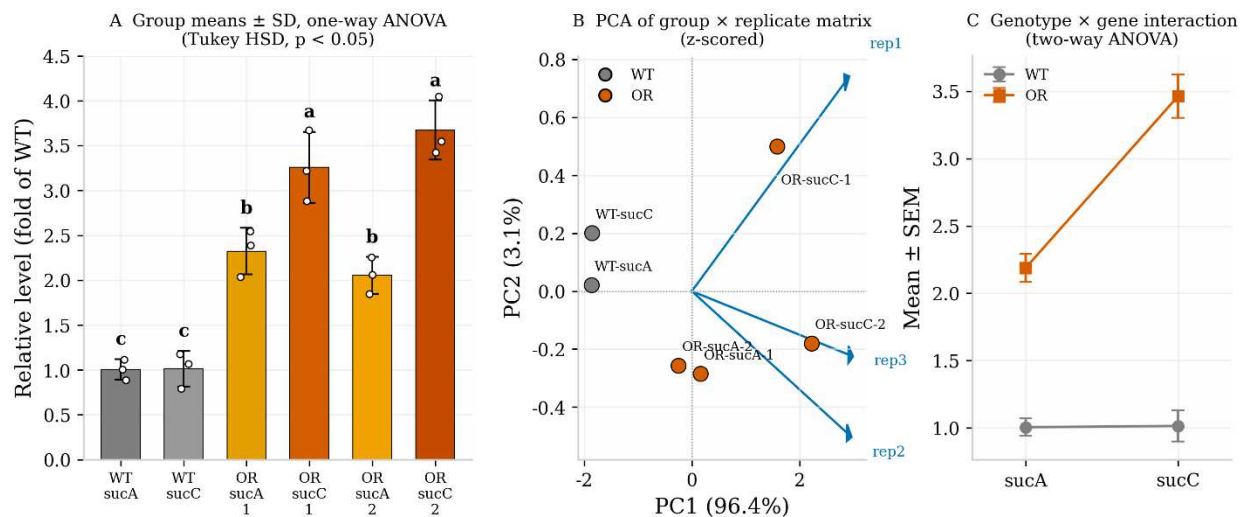
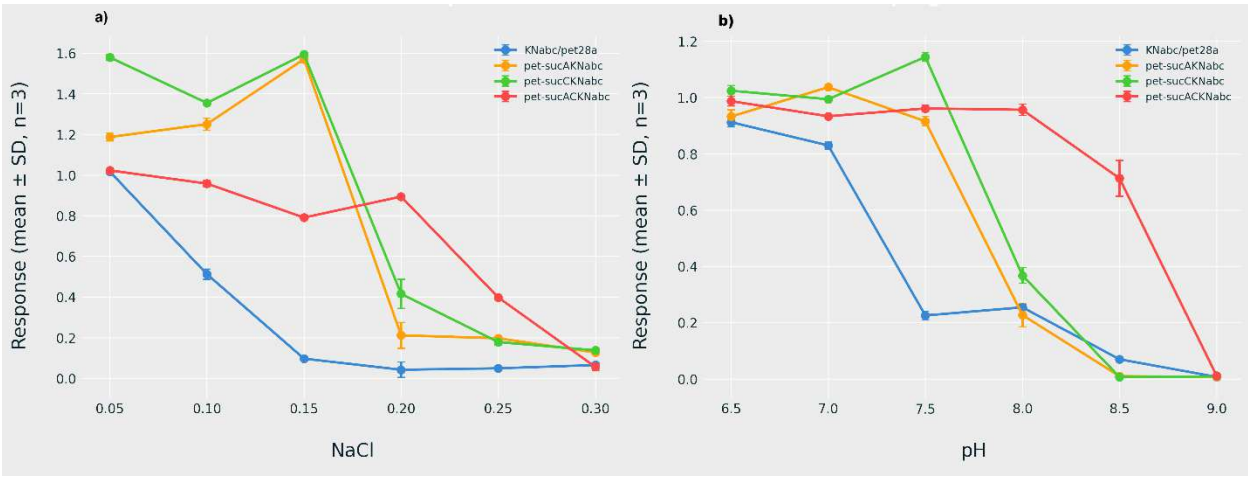


Figure 9. qRT-PCR quantitative analysis of *sucA* and *sucC* genes



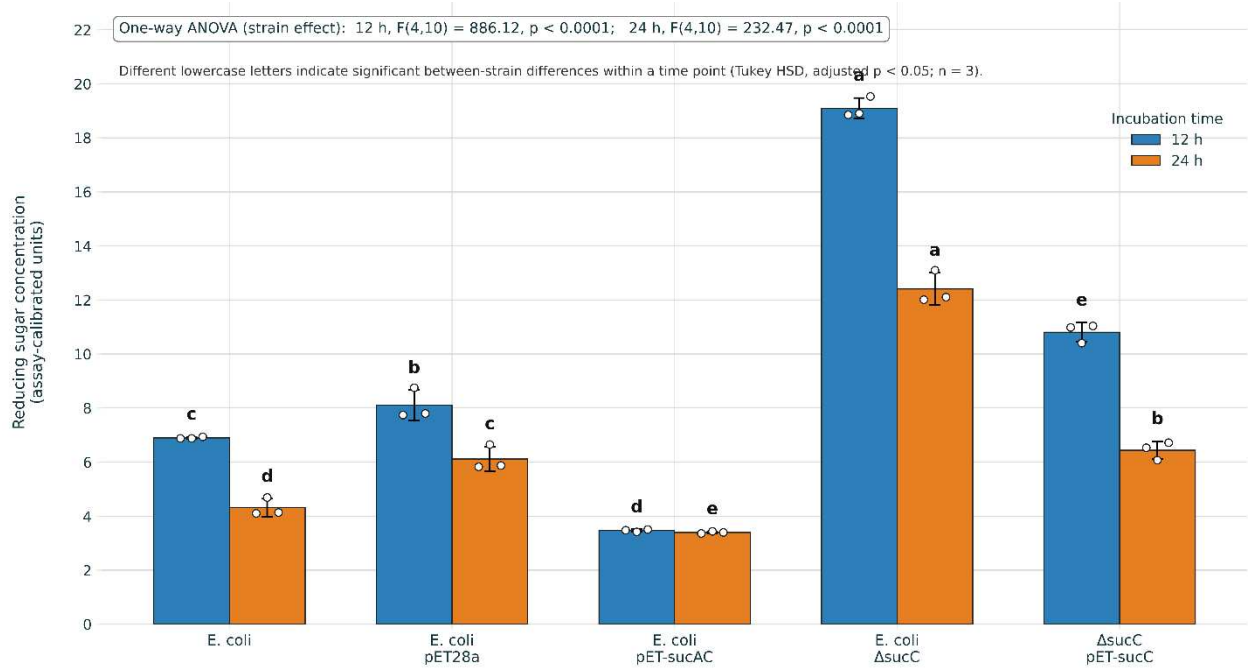
a) Relative expression levels of *sucA* and *sucC* in wild-type (WT) and overexpression (OE) strains, as determined by qRT-PCR. Bars represent mean $\pm$ SD; $p < 0.001$ by one-way ANOVA; b) PCA analysis; c) genotype $\times$ gene interaction.

Figure 10. Functional complementation of saline-alkali tolerance in for pET-*sucAC* in *E. coli* KNabc



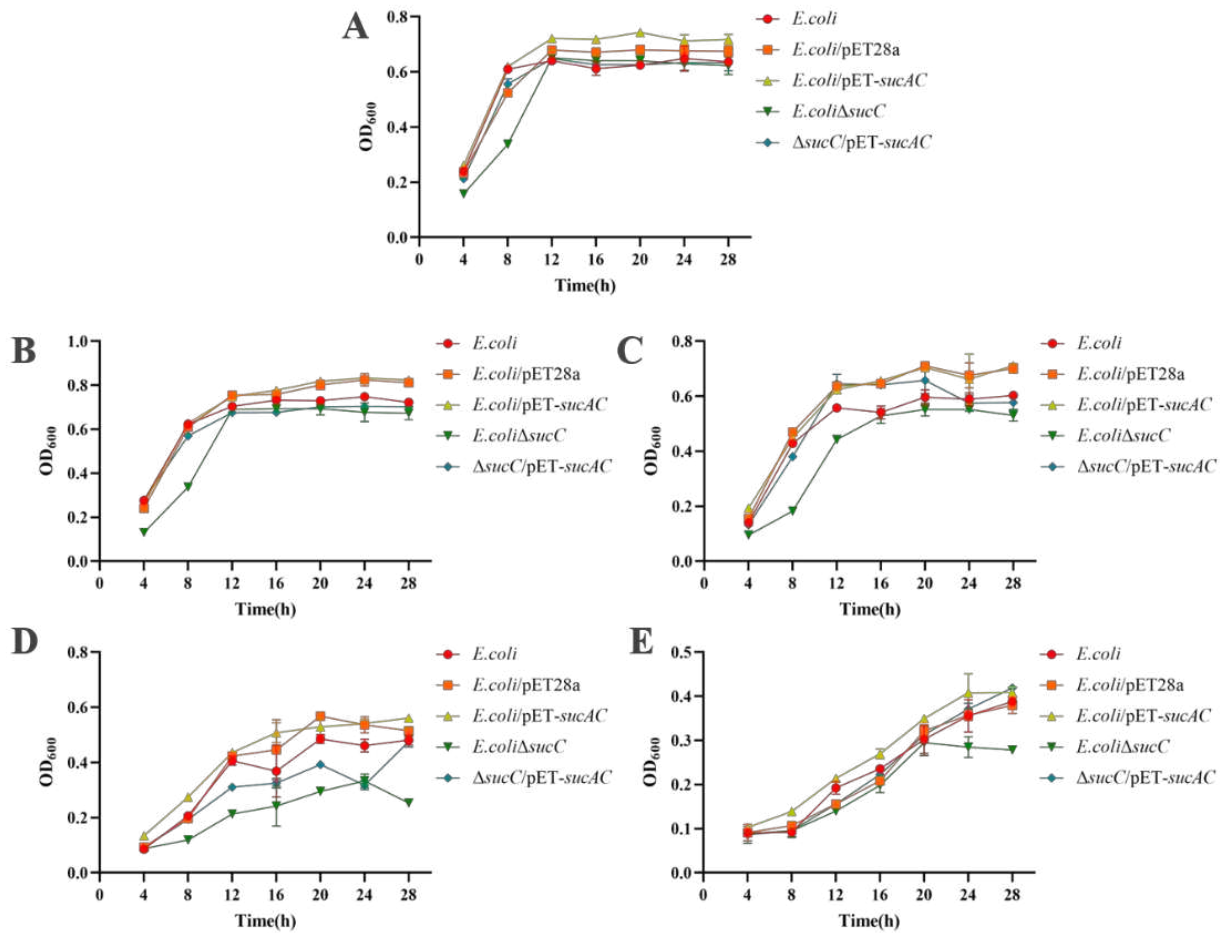
a) Growth of KNabc strains harboring pET28a (empty vector), pET-*sucA*, pET-*sucC*, or pET-*sucAC* across a NaCl gradient (0.05–0.3 M); **b)** Alkaline tolerance at pH 6.5–9.0 with 50 mM NaCl. Data represent mean $\pm$ SD from three replicates.

Figure 11. Metabolic profiling of reducing sugar measured by the DNS method



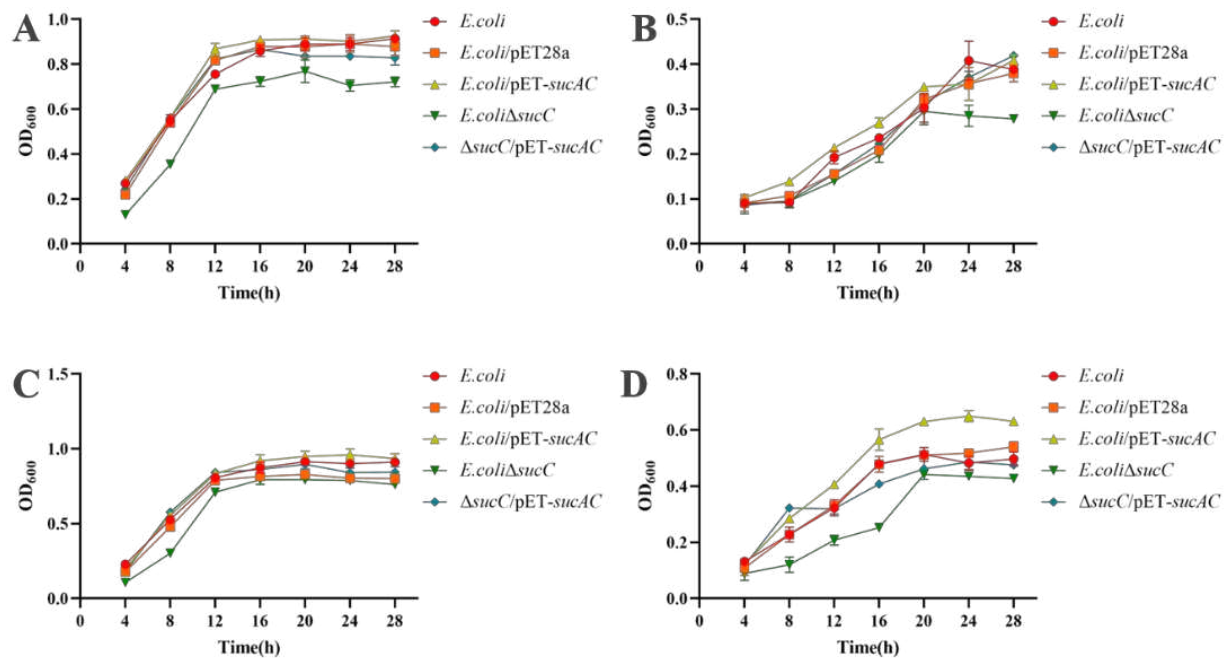
Different lowercase letters indicate significant differences (Tukey's HSD, $p < 0.05$).

Figure 12. Growth dynamics of engineered *E. coli* strains under salt stress



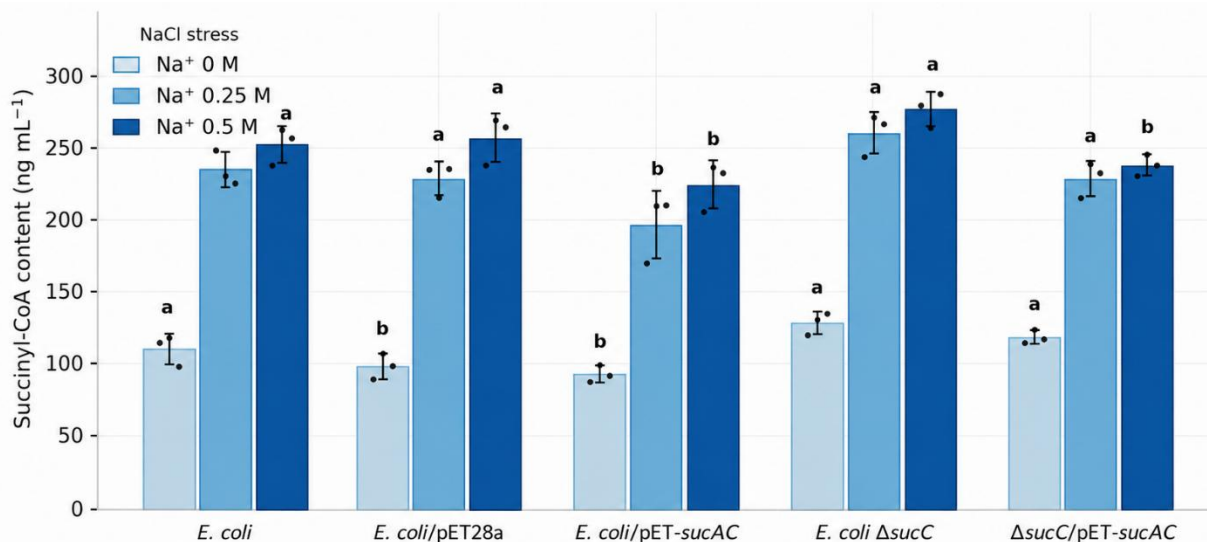
a-e) Time-course growth curves at 0, 0.25, 0.5, 0.75, and 1.0 M NaCl, respectively. Strains: wild-type *E. coli*(red); *E. coli*/pET28a (orange); *E. coli*/pET-sucAC (yellow); *E. coli*ΔsucC(green); ΔsucC/pET-sucAC (blue). Significance determined by one-way ANOVA ($p < 0.05$).

Figure 13. Growth dynamics of engineered *E. coli* strains under different alkaline stress concentrations



a) pH 8; b) pH 8.5; b) pH 9; d) pH 9.5 are the time-course growth curves. Strains: wild-type *E. coli* (red); *E. coli*/pET28a (orange); *E. coli*/pET-*sucAC* (yellow); *E. coli*Δ*sucC* (green); Δ*sucC*/pET-*sucAC* (blue). Significance determined by one-way ANOVA ($p < 0.05$).

Figure 14. Determination of metabolic profiling of reducing succinyl-CoA content



Metabolic profiling at 0, 0.25, 0.5, M Na⁺, respectively. Strains: wild-type *E. coli*; *E. coli*/pET28a; *E. coli*/pET-*sucAC*; *E. coli*Δ*sucC*; Δ*sucC*/pET-*sucAC*. Significance determined by one-way ANOVA ($p < 0.05$), different lowercase letters indicate significant differences (Tukey's HSD, $p < 0.05$).

Supplementary Files

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- [SupplementaryFigurelist27.9.2026.docx](#)