

Supplementary Material

High-yield production of (*R*)-acetoin in *Saccharomyces cerevisiae* by deleting genes for NAD(P)H-dependent ketone reductases producing meso-2,3-butanediol and 2,3-dimethylglycerate

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Table S1 Primers used in this study

Primers	Sequence (5'-3')
For cloning ^a	
ARA1 pRS NheI F	TCGAGCTAGCATGTCTTCTTCAGTAGCCTC
ARA1 pRS BamHI R	TCGAGGATCCTTAATACTTTAAATTGTCCAAGTTTG
YPR1 pRS SpeI F	TCGA <u>ACTAGT</u> ATGCCTGCTACGTAAAGAA
YPR1 pRS XhoI R	TCGACTCGAGTCATTGGAAAATTGGGAAGGA
ORA1 pRS BamHI F	TCGAGGATCCATGTCCCAAGGTAGAAAAGC
ORA1 pRS SalI F	TCGAGTCGACTTATCCACGGAAGATATGATGAG
ARA1 pET PstI F	TCGAACATGTCCTCTTCTTCAGTAGCCTCAAC
ARA1 pET NotI R	TCGAGCGGCCGCATACTTTAAATTGTCCAAGTTTGG
YPR1 pET NcoI F	TCGACCATGGGTCCTGCTACGTAAAGAATTC
YPR1 pET NotI R	TCGAGCGGCCGCTTGAAAATTGGGAAGGATC
ORA1 pET NcoI F	TCGACCATGGGTTCCCAAGGTAGAAAAGCTGC
ORA1 pET NotI R	TCGAGCGGCCGCTCCACGGAAGATATGATGAG
alsS pET NcoI F	TCGACCATGGGTACAAAAGCAACAAAAGAACA
alsS pET NotI R	TCGAGCGGCCGCGAGAGCTTTCGTTTTTCATGA
For generating gRNA ^b	
ARA1 target gRNA F	<u>TACGAATGGCTCTGTCTCGT</u> GTTTTAGAGCTAGAAATAGC
ARA1 target gRNA R	<u>ACGAGACAGAGCCATTCGT</u> AGATCATTTATCTTTCCTGC
BDH2 target gRNA F	<u>AAGGTAGTTGTGAGCCACG</u> TTTTAGAGCTAGAAATAGC
BDH2 target gRNA R	<u>GTGGGCTCGACAACCTT</u> GATCATTTATCTTTCCTGC
YPR1 target gRNA F	<u>ATGCAAGAGTTGCCAAAGAC</u> GTTTTAGAGCTAGAAATAGC
YPR1 target gRNA R	<u>GTCTTTGGCAACTCTTGCA</u> TGATCATTTATCTTTCCTGC
GRE3 target gRNA F	<u>TGGTTGTAGAATCAAGCCCG</u> TTTTAGAGCTAGAAATAGC
GRE3 target gRNA R	<u>CGGGCTTGATTCTACAACC</u> AGATCATTTATCTTTCCTGC

YJR096W target gRNA F	<u>AGAAGCGGTTGATGAAGGATGTTTTAGAGCTAGAAATAGC</u>
YJR096W target gRNA R	<u>ATCCTTCATCAACCGCTTCTGATCATTTATCTTTCAGTGC</u>
ORA1 target gRNA F	<u>AGTTAGATACAGAGGTAACGGTTTTAGAGCTAGAAATAGC</u>
ORA1 target gRNA R	<u>CGTTACCTCTGTATCTAACTGATCATTTATCTTTCAGTGC</u>
NRE1 target gRNA F	<u>AGGTATCGGTAAGTCCATCGGTTTTAGAGCTAGAAATAGC</u>
NRE1 target gRNA R	<u>CGATGGACTTACCGATACCTGATCATTTATCTTTCAGTGC</u>
IRC24 target gRNA F	<u>CGTCTACGGCGTAGCAAGAAAGTTTTAGAGCTAGAAATAGC</u>
IRC24 target gRNA R	<u>TTCTTGCTACGCCGTAGACGGATCATTTATCTTTCAGTGC</u>
ENV9 target gRNA F	<u>AGGAAGATTGCTGTAGTAACGTTTTAGAGCTAGAAATAGC</u>
ENV9 target gRNA R	<u>GTTACTACAGCAATCTTCCTGATCATTTATCTTTCAGTGC</u>
For deletion confirmation	
ARA1 upstream F	GCCTCCACCTTAACATCTTA
ARA1 downstream F	ACGTACGGCGAATGATTATA
BDH2 upstream F	GCATTGGTTAGCTCAGATAT
BDH2 downstream R	CTGCCCCACTTTTAT ATGTC
YPR1 upstream F	AGCCTATTTGGAAAAGACTG
YPR1 downstream R	CAGTAGAAGCGCAACTAGTA
GRE3 upstream F	TGTTTCCCAATTGTTGCTGG
GRE3 downstream R	TTGGGACCGCTTTGCTCTCT
YJR096W upstream F	TTGTCCTTATTTGAGGCTCC
YJR096W downstream R	ATTGCGCTTATCTTTTGCA
ORA1 upstream F	AACACTCGACCAGAACGATC
ORA1 downstream R	CAGCCTAGTTTAGCCAAATC
NRE1 upstream F	TCAATATCTCCGCTACAACG
NRE1 downstream R	GATGTAATGTGACGGCAGCC
IRC24 upstream F	TTCTTGTC AACAGGTGCTAG

IRC24 downstream R	TTACCGATACCTCTGGAAAC
ENV9 upstream F	ATTGAGCCACAGGTCTTTCG
ENV9 downstream R	AGATCCAAGCCTGATAGACC
For qPCR	
ACT1 F	GCCGAAAGAATGCAAAAGGA
ACT1 R	TAGAACCACCAATCCAGACGG
alsS F	CGCACCTCTTGAAATCGTT
alsS R	CCGAGTGTTTGCATACCGTT

^a Restriction enzyme sites are underlined.

^b Gene-specific gRNA sequences are underlined.

Table S2 Sequences of chromosomal integration site

Target	Sequence (5'-3')
ADH2	<p> CCACTTCACGAGACTGATCTCCTCTGCCGGAACACCGGGCATCTCCAACCTATAAGTTGGA GAAATAAGAGAATTTTCTAGATTGAGAGAATGAAAAAAAAAAAAAAAAAAGGCAGAGGAGA GCATAGAAATGGGGTTCACTTTTTGGTAAAGCTATAGCATGCCTATCACATATAAATAGAG TGCCAGTAGCGACTTTTTTTCACACTCGAAATACTCTTACTACTGCTCTCTTGTTGTTTTTA TCACTTCTTGTTTCTTCTTGGTAAATAGAATATCAAGCTACAAAAAGCATACAATCAACTA TCAACTATTAATACTATATCGTAATACACAATGTCTATTCCAGAAACTCAAAAAGCCATTATC TTCTACGAATCCAACGGCAAGTTGGAGCATAAGGATATCCCAGTTCCAAAGCCAAAGCCCA ACGAATTGTTAATCAACGTCAGTACTCTGGTGTCTGCCACACCGATTTGCACGCTTGGCA TGGTGACTGGCCATTGCCAACTAAGTTACCATTAGTTGGTGGTTCACGAAGGTGCCGGTGTG GTTGTCCGCATGGGTGAAAACGTTAAGGGCTGGAAGATCGGTGACTACGCCGGTATCAAAT GGTTGAACGGTCTTGTATGGCCTGTGAATACTGTGAATTGGGTAAACGAATCCAACGTGTCC TCACGCTGACTTGTCTGGTTACACCCACGACGGTTCTTTCCAAGAATACGCTACCGCTGAC GCTGTTCAAGCCGCTCACATTCTCAAGGTACTGACTTGGCTGAAGTCGCGCCAATCTTGT GTGCTGGTATCACCGTATACAAGGCTTTGAAGTCTGCCAACTTGAGAGCAGGCCACTGGGC GGCCATTTCTGGTGCTGCTGGTGGTCTAGGTTCTTTGGCTGTTCAATATGCTAAGGCGATG GGTTACAGAGTCTTAGGTATTGATGGTGGTCCAGGAAAGGAAGAATTGTTTACCTCGCTCG GTGGTGAAGTATTCATCGACTTCACCAAAGAGAAGGACATTGTTAGCGCAGTCGTTAAGGC TACCAACGGCGGTGCCACGGTATCATCAATGTTTCCGTTTCCGAAGCCGCTATCGAAGCT TCTACCAGATACTGTAGGGCGAACGGTACTGTTGTCTTGGTTGGTTTGCCAGCCGGTGCAA AGTGCTCCTCTGATGTCTTCAACCACGTTGTCAAGTCTATCTCCATTGTCTGGCTCTTACGT GGGGAACAGAGCTGATACCAGAGAAGCCTTAGATTTCTTTGCCAGAGGTCTAGTCAAGTCT CCAATAAAAGGTAGTTGGCTTATCCAGTTTACCAGAAATTTACGAAAAGATGGAGAAGGGCC AAATTGCTGGTAGATACGTTGTTGACACTTCTAAATAAGCGGATCTCTTATGTCTTTACGA TTTATAGTTTTTCAATTATCAAGTATGCCTATATTAGTATATAGCATCTTTAGATGACAGTGT TCGAAGTTTACGAATAAAAGATAATATTCTACTTTTTGCTCCACCGCGTTTGCTAGCAC GAGTGAACACCATCCCTCGCCTGTGAGTTGTACCCATTCTCTAACTGTAGACATGGTAG CTTCAGCAGTGTTTCGTTATGTACGGCATCCTCCAACAAACAGTCGGTTATAGTTTGTCTG CTCCTCTGAATCGTCTCCCTCGATATTTCTCATTT </p>
YARCdelta4	<p> TGTTGGAATAGAAATCAACTATCATCTACTAACTAGTATTTACATTACTAGTATATTATCA TATACGGTGTTAGAAGATGACGCAAATGATGAGAAATAGTCATCTAAATTAGTGGAAGCTG AAACGCAAGGATTGATAATGTAATAGGATCAATGAATATAAACATATAAAACGGAATGAGG AATAATCGTAATATTAGTATGTAGAAATATAGATTCCATTTTGAGGATTCCTATATCCTCG AGGAGAACTTCTAGTATATTCTGTATACCTAATATTATAGCCTTTATCAACAATGGAATCC CAACAATTATCTCAACATTCACCCATTTCTCA </p>

ORF sequences were described italics.

5' or 3' flanking region of gene expression cassettes were described red or blue, respectively.

Table S3 Kinetic parameters of Ara1, Ypr1, and Ymr226C

	V_{\max} (mM·min ⁻¹)	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m
Ara1	0.023	0.203	0.909	4.480
Ypr1	0.017	0.124	0.690	5.561
Ora1	0.026	0.205	1.042	5.070

Supplementary methods

Estimation of gene copy numbers integrated into genome

To determine the copy numbers of the delta-integrated gene expression cassette, quantitative PCR (qPCR) was performed. The reaction mixture containing 5 ng genomic DNA, SYBR Green I master mix (Roche Applied Science) and 5 pmol each of gene-specific primers were amplified with 45 cycles under the condition of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s using the LightCycler 480 II system (Roche Applied Science). The crossing point (Cp) values were determined using the LightCycler 480 software version 4.5 and relative expression levels were represented as normalized target/reference ratios ($[alsS/ACT1]$).

Analysis of enzyme kinetics

Enzyme activities of Ara1, Ypr1, and Ora1 were determined by measuring the oxidation rate of NADH. Each 25 μ M protein was incubated with various concentrations of (R/S)-acetoin (31.25, 62.5, 125, 250, and 500 μ M), 500 μ M NADH in 50 mM Tris-HCl (pH 7.0) buffer at 30°C. The reaction was initiated by adding each protein, and the change of absorbance at 340 nm was monitored with 1 min intervals using microplate reader (SynergyTM H1, Biotek). The kinetic parameters of Ara1, Ypr1, and Ora1 were determined from a Lineweaver-Burk plot by fitting the initial velocity (within 5 min).

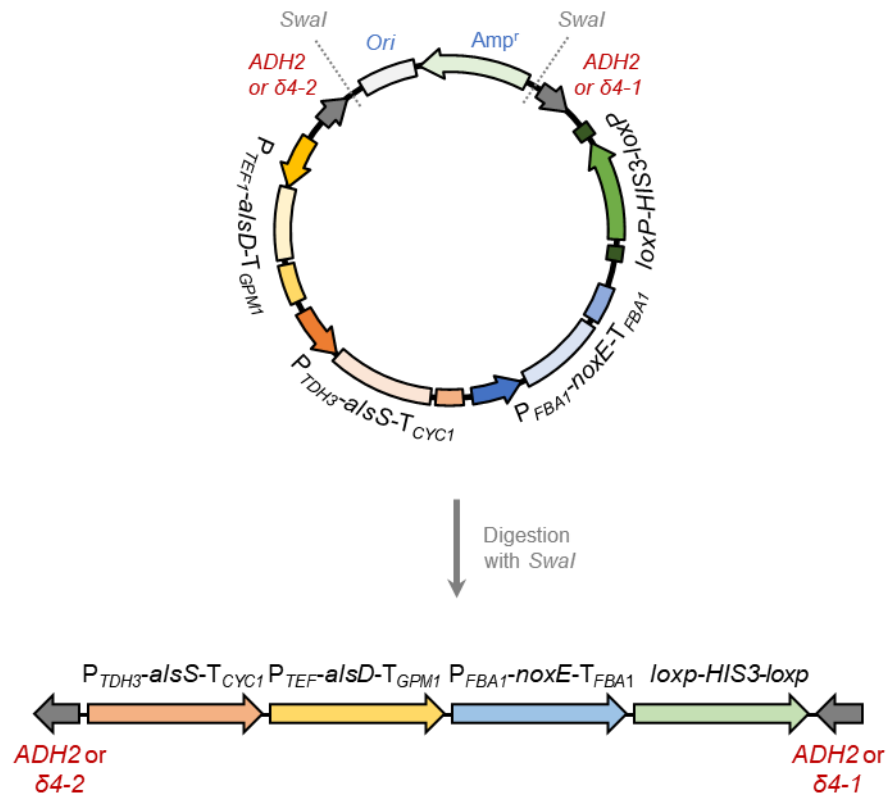


Figure S1. Schematic illustration of the construction of an acetoin pathway-integration cassette. The integrative plasmid consists of gene expression cassettes (black characters), homologous sequences of target site (red characters), and components for cloning (blue characters). After digesting the integrative plasmid with *SwaI*, the resulting mixtures of DNA fragments were introduced into yeast cells and selected on SC-His medium. The experimental details are described in materials and methods.

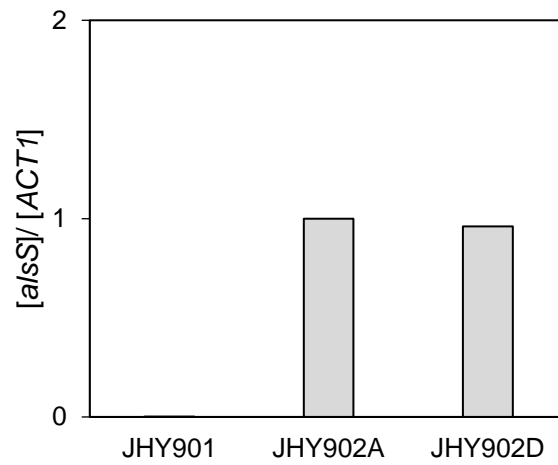
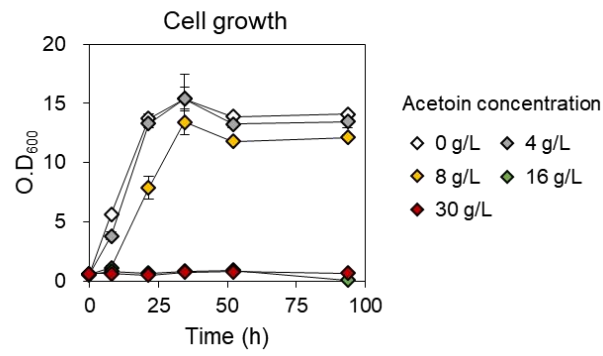


Figure S2. Copy numbers of the gene expression cassette integrated into delta sequences of JHY902D strain. The *alsS* copy number was quantified by qPCR and normalized to the value obtained from JHY902A strain where one copy of *alsS* gene is integrated at the *ADH2* locus. JHY901 strain was used as negative control.

A



B

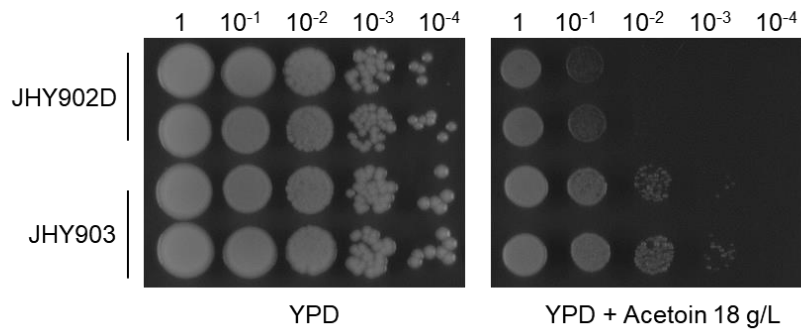


Figure S3. Acetoin tolerance of JHY902D and JHY903 strains. A) The JHY902D cells were grown in YPD medium and diluted to OD₆₀₀ of 0.5 in YPD medium containing 0, 4, 8, 16, 30 g/L acetoin. B) The JHY902D and JHY903 cells were grown in YPD medium, and OD₆₀₀ of 1 cells were serially diluted and spotted onto YPD solid medium with or without 18 g/L acetoin.

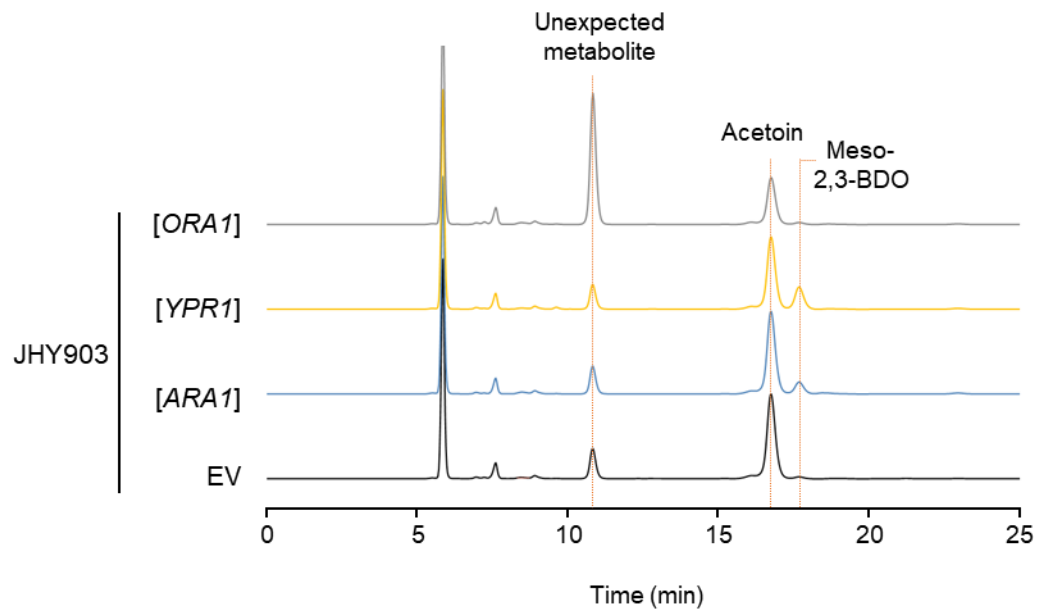
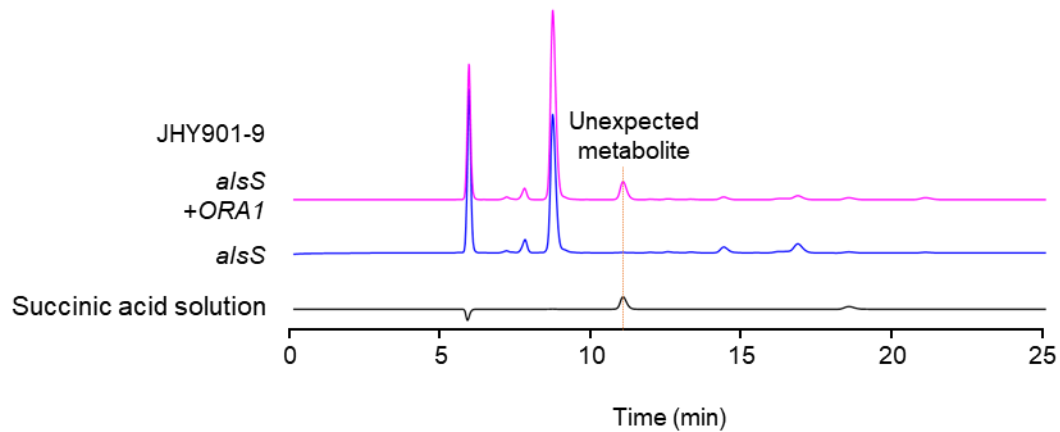
A**B**

Figure S4. Analysis of fermentation broth on the HPX-87H column. HPLC peaks of fermentation products from (A) JHY903 cells harboring p413GPD, p414GPD, and p416GPD (JHY903[EV]) or harboring p416G-ARA1 (JHY903[*ARA1*]), p416G-YPR1 (JHY903[*YPR1*]) or p416G-ORA1 (JHY903[*ORA1*]) together with p413GPD and p414GPD after 48h (B) JHY901-9 cells harboring p413A-*alsS*, p414GPD, and p416GPD or harboring p413A-*alsS*, p414GPD, and p416G-*ORA1*.

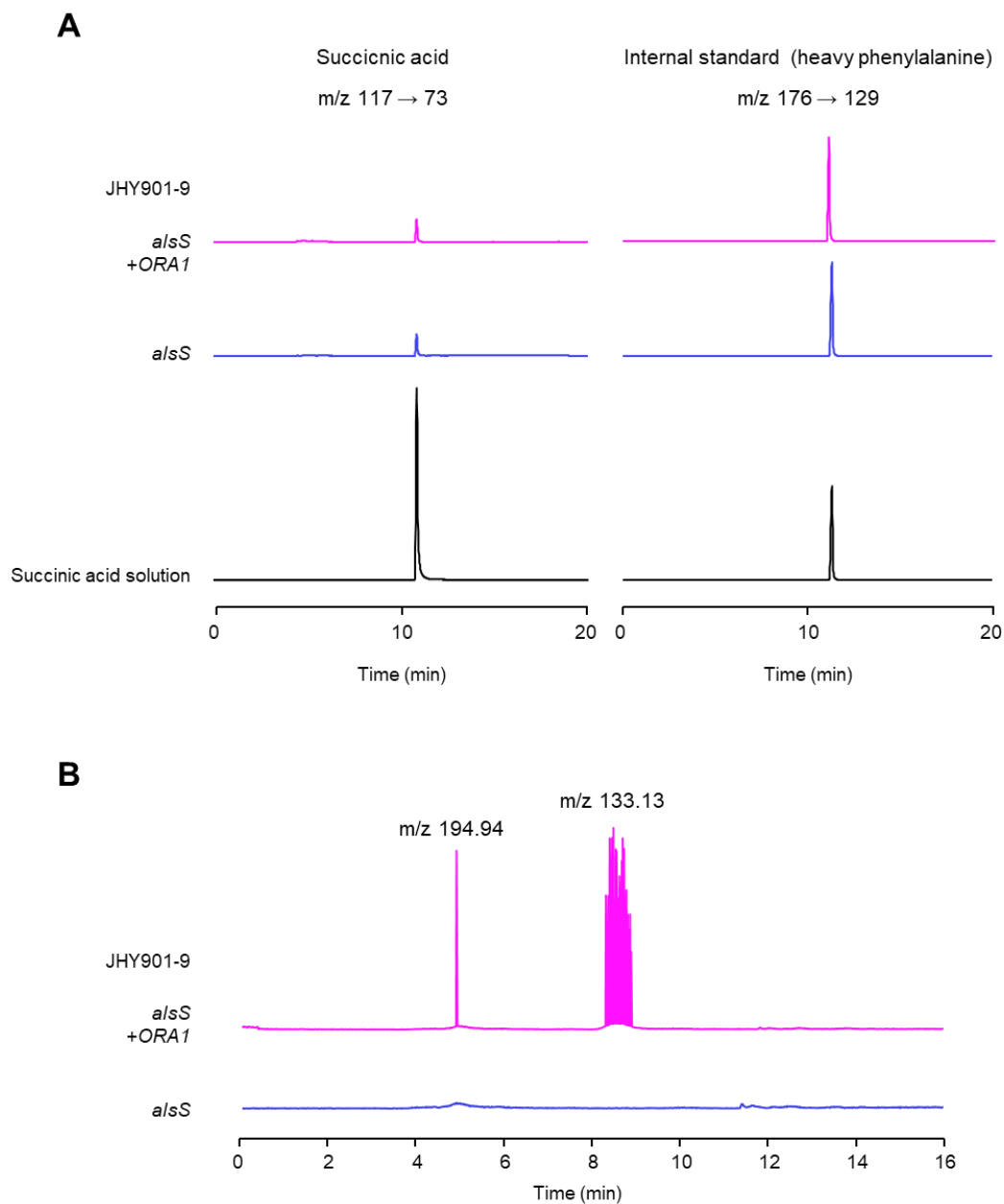


Figure S5. Analysis of metabolites in JHY901-9 strain overexpressing *alsS* or *alsS* and *ORA1*. (A) Full LC-MS spectra using C18 Hypersil GOLD column (B) Monitoring of succinate using LC-MS/MS with SRM scan mode ([M-H]⁻ m/z 117.1 → 73.3). Heavy phenylalanine was used as an internal standard.

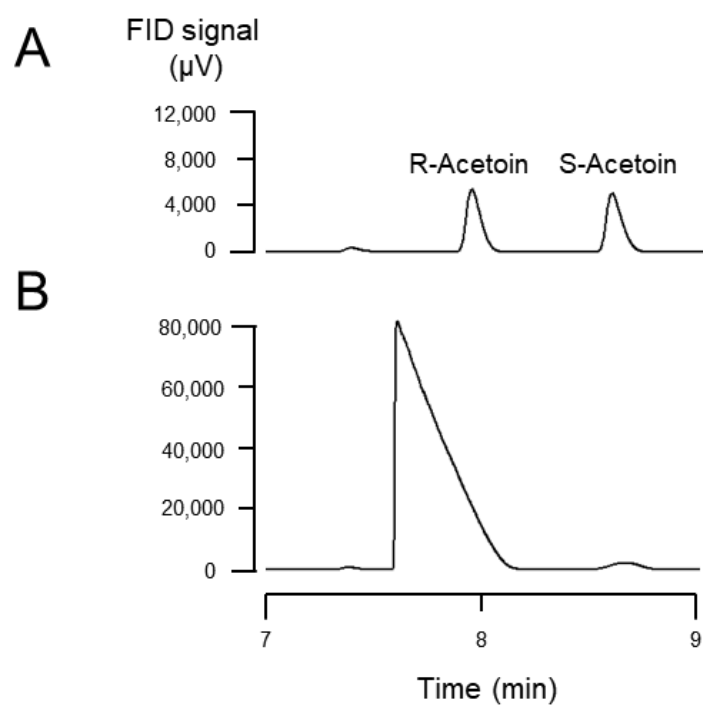


Figure S6. Identification of acetoin stereoisomers by gas chromatography (GC) analysis. (A) HPLC peaks of (*R/S*)-acetoin (racemic mixture) standard solution. (B) HPLC peaks of fed-batch fermentation products of JHY903-159 strain after 86 h cultivation.