Construction and application of an efficient dual-base editing platform for *Bacillus subtilis* evolution employing programmable base conversion

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Supplementary Information

Table S1. The strains used in this study

Strains	Description	References or sources
E. coli JM109	*recA*1, *supE*44 *endA*1 *hsdR*17 (r^{-}k,m^{+}k) *gyrA*96 *relA*1 thi (*lac-proAB*) F'[*traD*36 *proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15]	Lab stock
B. subtilis 168	*trpC*2	Lab stock
BS1	Derived from *B. subtilis* 168, *lacA*::P_{xylA}-ABE7.10-CDA-nCas9, Cm^{R}	This study
BS2	Derived from *B. subtilis* 168, *lacA*::P_{xylA}-CDA-ABE7.10-nCas9, Cm^{R}	This study
BS3	Derived from *B. subtilis* 168, *lacA*::P_{xylA}-ABE7.10-nCas9-CDA , Cm^{R}	This study
BS4	Derived from *B. subtilis* 168, *lacA*::P_{xylA}-ABE8e-CDA-nCas9, Cm^{R}	This study
BS5	Derived from *B. subtilis* 168, *lacA*::P_{xylA}-CDA-ABE8e-nCas9, Cm^{R}	This study
BS6	Derived from BS4, *amyE*::P_{veg}-B1/P_{veg}-B2/P_{veg}-B3/P_{veg}-B4/P_{veg}-B5/P_{veg}-B6/P_{veg}-B7, Cm^{R}, Spc^{R}	This study
BS7	Derived from BS4, *amyE*::P_{veg}-B2/P_{veg}-B3/P_{veg}-B4, Cm^{R}, Spc^{R}	This study
BS8	Derived from BS4, *amyE*::P_{veg}-P1/P_{veg}-P2/P_{veg}-	This study

	P3/P_{veg}-P4/P_{veg}-P5, Cm^R, Spc^R	
BS9	Derived from BS4, *amyE*::P_{veg}-P1/P_{veg}-P5, Cm^R, Spc^R	This study
BS4-pJOE-B2	Derived from BS4, harboring pJOE-B2 plasmid, Kan^R, Cm^R	This study
BS4-pJOE-B3	Derived from BS4, harboring pJOE-B3 plasmid, Kan^R, Cm^R	This study
BS4-pJOE-B4	Derived from BS4, harboring pJOE-B4 plasmid, Kan^R, Cm^R	This study

Table S2. Primers used in this study

Primers	Sequences (5'-3')
pHY-B1-F	cgcaacaaacagttcattaagttttagagctagaaatagcaagttaaaataag
pHY-B1-R	ttaatgaactgtttgttgcgtttatattttacataatcgcgcgc
pHY-B2-F	agccgctacattttgttcaggttttagagctagaaatagcaagttaaaataag
pHY-B2-R	ctgaacaaaatgtagcggcttttatattttacataatcgcgcgc
pHY-B3-F	tcaacatttgcttgcaggacgttttagagctagaaatagcaagttaaaataag
pHY-B3-R	gtcctgcaagcaaatgttgatttatattttacataatcgcgcgc
pHY-B4-F	acagcaagctgcatattgccgttttagagctagaaatagcaagttaaaataag
pHY-B4-R	ggcaatatgcagcttgctgttttatattttacataatcgcgcgc
pHY-B5-F	gcccccgcttgtaaacgtgtgttttagagctagaaatagcaagttaaaataag
pHY-B5-R	acacgtttacaagcgggggctttatattttacataatcgcgcgc
pHY-B6-F	cacccatttgtttaaaataagttttagagctagaaatagcaagttaaaataag
pHY-B6-R	ttattttaaacaaatgggtgtttatattttacataatcgcgcgc
pHY-B7-F	aacaccatcaccataatcatgttttagagctagaaatagcaagttaaaataag
pHY-B7-R	atgattatggtgatggtgtttttatattttacataatcgcgcgc
pHY-pksA1-F	atcagccagaaatattgcaagttttagagctagaaatagcaagttaaaataag
pHY-pksA1-R	ttgcaatatttctggctgattttatattttacataatcgcgcgc
pHY-pksA2-F	cacgcgcacagggtcaagcagttttagagctagaaatagcaagttaaaataag
pHY-pksA2-R	tgcttgaccctgtgcgcgtgtttatattttacataatcgcgcgc
pHY-pksB-F	cattcaaagaataaagtcgggttttagagctagaaatagcaagttaaaataag
pHY-pksB-R	ccgactttattctttgaatgtttatattttacataatcgcgcgc
pHY-pksC-F	tcatcgcataaagaaagatggttttagagctagaaatagcaagttaaaataag

pHY-pksC-R	catctttctttatgcgatgatttatattttacataatcgcgcgc
pHY-pksD1-F	gttcccaatactatcacatggttttagagctagaaatagcaagttaaaataag
pHY-pksD1-R	catgtgatagtattgggaactttatattttacataatcgcgcgc
pHY-pksD2-F	actcaatcataaaaatggcggttttagagctagaaatagcaagttaaaataag
pHY-pksD2-R	cgccatttttatgattgagttttatattttacataatcgcgcgc
pHY-pksE1-F	acacaaatatcgtcagccatgttttagagctagaaatagcaagttaaaataag
pHY-pksE1-R	atggctgacgatatttgtgttttatattttacataatcgcgcgc
pHY-pksE2-F	tcccgcggcagacattgagagttttagagctagaaatagcaagttaaaataag
pHY-pksE2-R	tctcaatgtctgccgcgggatttatattttacataatcgcgcgc
pHY-pksF-F	cacgggattagcatatacgggttttagagctagaaatagcaagttaaaataag
pHY-pksF-R	ccgtatatgctaatcccgtgtttatattttacataatcgcgcgc
pHY-pksG-F	gcaccggacaatgatgcgtagttttagagctagaaatagcaagttaaaataag
pHY-pksG-R	tacgcatcattgtccggtgctttatattttacataatcgcgcgc
ABE7.10-CDA-nCas9-b-F	tggaggaagcacagatgccgaatacgttcg
ABE7.10-CDA-nCas9-b-R	aacttcgctcatggatcccatttccccctttgattttta
TadA1-F	ggaaatgggatccatgagcgaagttgaattcagcc
TadA1-R	cggcatctgtgcttcctccagatgagcctc
ABE8e-CDA-nCas9-b-F	tggaggaagcacagatgccgaatacgttcg
ABE8e-CDA-nCas9-b-R	cttctgacatggatcccatttccccctttgattttta
ABE8e1-F	aatgggatccatgtcagaagttgaattttcacatg
ABE8e1-R	cggcatctgtgcttcctccagatgagcctc
CDA-ABE7.10-nCas9-b-F	tctggaggaagcgataagaaatactcaataggcttagctatcg
CDA-ABE7.10-nCas9-b-R	caacttcgctagactctggagttgcagactc
TadA2-F	tccagagtctagcgaagttgaattcagccac
TadA2-R	gagtatttcttatcgcttcctccagatgagcctc
CDA-ABE8e-nCas9-b-F	atcactcacacgcataaagtgttttagagctagaaatagcaagttaaaataag
CDA-ABE8e-nCas9-b-R	actttatgcgtgtgagtgattttatattttacataatcgcgcgc
ABE8e2-F	cctgcaggattcactttcaagttttagagctagaaatagcaagttaaaataag
ABE8e2-R	ttgaaagtgaatcctgcaggtttatattttacataatcgcgcgc
ABE7.10-nCas9-CDA-b-F	tctggaggaagcgataagaaatactcaataggcttagctatcg
ABE7.10-nCas9-CDA-b-R	aacttcgctcatggatcccatttccccctttgattttta
TadA3-F	ggaaatgggatccatgagcgaagttgaattcagcc
TadA3-R	gagtatttcttatcgcttcctccagatgagcctc

bceB-B1-F	cgcaacaaacagttcattaagttttagagctagaaatagcaagttaaaataag
bceB-B1-R	ttaatgaactgtttgttgcgacatttattgtacaacacgagcc
bceB-B2-F	agccgctacattttgttcaggttttagagctagaaatagcaagttaaaataag
bceB-B2-R	ctgaacaaaatgtagcggctacatttattgtacaacacgagcc
bceB-B3-F	tcaacatttgcttgcaggacgttttagagctagaaatagcaagttaaaataag
bceB-B3-R	gtcctgcaagcaaatgttgaacatttattgtacaacacgagcc
bceB-B4-F	acagcaagctgcatattgccgttttagagctagaaatagcaagttaaaataag
bceB-B4-R	ggcaatatgcagcttgctgtacatttattgtacaacacgagcc
bceB-B5-F	gcccccgcttgtaaacgtgtgttttagagctagaaatagcaagttaaaataag
bceB-B5-R	acacgtttacaagcgggggcacatttattgtacaacacgagcc
bceB-B6-F	cacccatttgtttaaaataagttttagagctagaaatagcaagttaaaataag
bceB-B6-R	ttattttaaacaaatgggtgacatttattgtacaacacgagcc
bceB-B7-F	aacaccatcaccataatcatgttttagagctagaaatagcaagttaaaataag
bceB-B7-R	atgattatggtgatggtgttacatttattgtacaacacgagcc
psdB-P1-F	aaacacacagcttaaaaagtgttttagagctagaaatagcaagttaaaataag
psdB-P1-R	acttttaagctgtgtgtttacatttattgtacaacacgagcc
psdB-P2-F	cgctcaagcatgttttgacagttttagagctagaaatagcaagttaaaataag
psdB-P2-R	tgtcaaaacatgcttgagcgacatttattgtacaacacgagcc
psdB-P3-F	taacaggcctgctccaatgcgttttagagctagaaatagcaagttaaaataag
psdB-P3-R	gcattggagcaggcctgttaacatttattgtacaacacgagcc
psdB-P4-F	ctgaaacaggatgagaaagcgttttagagctagaaatagcaagttaaaataag
psdB-P4-R	gctttctcatcctgtttcagacatttattgtacaacacgagcc
psdB-P5-F	attaacactaaaatcaaaatgttttagagctagaaatagcaagttaaaataag
psdB-P5-R	attttgattttagtgttaatacatttattgtacaacacgagcc
B1-Go-F	ggtctcaatgcttattaacgttgatataatttaaattttatttgacaaaaatgg
B1-Go-R	ggtctcgaagtaaaaaagcaccgactcgg
B2-Go-F	ggtctcatccattattaacgttgatataatttaaattttatttgacaaaaatgg
B2-Go-R	ggtctcgcaccaaaaaagcaccgactcgg
B3-Go-F	ggtctcaggtgttattaacgttgatataatttaaattttatttgacaaaaatgg
B3-Go-R	ggtctcgatggaaaaaagcaccgactcgg
B4-Go-F	ggtctcaccatttattaacgttgatataatttaaattttatttgacaaaaatgg
B4-Go-R	ggtctcgagccaaaaaagcaccgactcgg
B5-Go-F	ggtctcaacttttattaacgttgatataatttaaattttatttgacaaaaatgg
B5-Go-R	ggtctcggctaaaaaaagcaccgactcgg
B6-Go-F	ggtctcaggctttattaacgttgatataatttaaattttatttgacaaaaatgg
B6-Go-R	ggtctcggcataaaaaagcaccgactcgg
B7-Go-F	ggtctcatagcttattaacgttgatataatttaaattttatttgacaaaaatgg
B7-Go-R	ggtctcgaagcaaaaaagcaccgactcgg
bceB-Go-b-F	ggtctcagcttaagcttgggcttaattaattaagactc
bceB-Go-b-R	ggtctcttggaggatcccgtcgacgc
P1-Go-F	ggtctcatccattattaacgttgatataatttaaattttatttgacaaaaatgg
P1-Go-R	ggtctcgcaccaaaaaagcaccgactcgg
P2-Go-F	ggtctcaggtgttattaacgttgatataatttaaattttatttgacaaaaatgg

P2-Go-R	ggtctcgatggaaaaaagcaccgactcgg
P3-Go-F	ggtctcaccatttattaacgttgatataatttaaattttatttgacaaaaatgg
P3-Go-R	ggtctcgagccaaaaaagcaccgactcgg
P4-Go-F	ggtctcaggctttattaacgttgatataatttaaattttatttgacaaaaatgg
P4-Go-R	ggtctcggcataaaaaagcaccgactcgg
P5-Go-F	ggtctcaatgcttattaacgttgatataatttaaattttatttgacaaaaatgg
P5-Go-R	ggtctcgaagcaaaaaagcaccgactcgg
psdB-Go-b-F	ggtctcagcttaagcttgggcttaattaattaagactc
psdB-Go-b-R	ggtctcttggaggatcccgtcgacgc
pJOE-F	atgcgaattcttattaacgttgatataatttaaattttatttgacaaaaatgg
pJOE-R	attattaaaaaaaaaaagcaccgactcgg
pJOE-b-F	gtgctttttttttaataatccaaatgagataaaaatgtcatgacattg
pJOE-b-R	acgttaataagaattcgcatcacacgcaaaaag

Table S3. The plasmids used in this study

Plasmids	Description	References or sources
pHYT-P43-G10	*E. coli-B. subtilis* shuttle vector, P43 promoter, gRNA targeting GFP, Tet^R	Lu et al., 2019[1]
pHY-ECBE	Derived from pHYT-P43-G10, gRNA targeting *sigE*	Lab stock
pHY-B1	Derived from pHYT-P43-G10, gRNA targeting *bceB* (B1)	This study
pHY-B2	Derived from pHYT-P43-G10, gRNA targeting *bceB* (B2)	This study
pHY-B3	Derived from pHYT-P43-G10, gRNA targeting *bceB* (B3)	This study
pHY-B4	Derived from pHYT-P43-G10, gRNA targeting *bceB* (B4)	This study
pHY-B5	Derived from pHYT-P43-G10, gRNA targeting *bceB* (B5)	This study
pHY-B6	Derived from pHYT-P43-G10, gRNA targeting *bceB* (B6)	This study
pHY-B7	Derived from pHYT-P43-G10, gRNA targeting *bceB* (B7)	This study
pHY-pksA-1	Derived from pHYT-P43-G10, gRNA targeting *pksA* (*pksA-1*)	This study
pHY-pksA-2	Derived from pHYT-P43-G10, gRNA targeting *pksA* (*pksA-2*)	This study
pHY-pksB	Derived from pHYT-P43-G10,	This study

	gRNA targeting *pksB* (*pksB*)	
pHY-pksC	Derived from pHYT-P43-G10, gRNA targeting *pksC* (*pksC*)	This study
pHY-pksD-1	Derived from pHYT-P43-G10, gRNA targeting *pksD* (*pksD-1*)	This study
pHY-pksD-2	Derived from pHYT-P43-G10, gRNA targeting *pksD* (*pksD-2*)	This study
pHY-pksE-1	Derived from pHYT-P43-G10, gRNA targeting *pksE* (*pksE-1*)	This study
pHY-pksE-2	Derived from pHYT-P43-G10, gRNA targeting *pksE* (*pksE-2*)	This study
pHY-pksF	Derived from pHYT-P43-G10, gRNA targeting *pksF* (*pksF*)	This study
pHY-pksG	Derived from pHYT-P43-G10, gRNA targeting *pksG* (*pksG*)	This study
pAD123	*E. coli-Bacillus* shuttle plasmid, promoter-less gfpmut3, Cm^R in *Bacillus*	Lab stock
pAD-B1	Derived from pAD123, gRNA targeting *bceB* (B1)	This study
pAD-B2	Derived from pAD123, gRNA targeting *bceB* (B2)	This study
pAD-B3	Derived from pAD123, gRNA targeting *bceB* (B3)	This study
pAD-B4	Derived from pAD123, gRNA targeting *bceB* (B4)	This study
pAD-B5	Derived from pAD123, gRNA targeting *bceB* (B5)	This study
pAD-B6	Derived from pAD123, gRNA targeting *bceB* (B6)	This study
pAD-B7	Derived from pAD123, gRNA targeting *bceB* (B7)	This study
pAD-P1	Derived from pAD123, gRNA targeting *psdB* (P1)	This study
pAD-P2	Derived from pAD123, gRNA targeting *psdB* (P2)	This study
pAD-P3	Derived from pAD123, gRNA targeting *psdB* (P3)	This study
pAD-P4	Derived from pAD123, gRNA targeting *psdB* (P4)	This study
pAD-P5	Derived from pAD123, gRNA targeting *psdB* (P5)	This study
pDGT-GFP	*B. subtilis* integration vector, P43-GFP cassette，$spec^R$	Lu et al., 2019 [1]

pDGT-GFP-Amp_m	Derived from pDGT-GFP, P43-GFP cassette，synonymous mutation in AmpR (TCT240TCC), $spec^R$	Lab stock
pDGT-B2-4	pDGT-GFP-Amp_m derivative, P_{veg} promoter, containing gRNA expression cassette targeting *bceB* (B2, B3, and B4)	This study
pDGT-B1-7	pDGT-GFP-Amp_m derivative, P_{veg} promoter, containing gRNA expression cassette targeting *bceB* (B1, B2, B3, B4, B5, B6, and B7)	This study
pDGT-P1-5	pDGT-GFP-Amp_m derivative, P_{veg} promoter, containing gRNA expression cassette targeting *psdB* (P1, P2, P3, P4, and P5)	This study
pDGT-P1-P5	pDGT-GFP-Amp_m derivative, P_{veg} promoter, containing gRNA expression cassette targeting *psdB* (P1 and P5)	This study
pAX-CDA-nCas9	*B. subtilis* integration vector, P_{xylA}-CDA-nCas9 expression cassette, $spec^R$，Cm^R	Lab stock
pAX-ABE7.10-CDA-nCas9	pAX-CDA-nCas9 derivative, P_{xylA}-ABE7.10-CDA-nCas9 expression cassette, $spec^R$，Cm^R	This study
pAX-ABE8e-CDA-nCas9	pAX-CDA-nCas9 derivative, P_{xylA}-ABE8e-CDA-nCas9 expression cassette, $spec^R$，Cm^R	This study
pAX-CDA-ABE7.10-nCas9	pAX-CDA-nCas9 derivative, P_{xylA}-CDA-ABE7.10-nCas9 expression cassette, $spec^R$，Cm^R	This study
pAX-CDA-ABE8e-nCas9	pAX-CDA-nCas9 derivative, P_{xylA}-CDA-ABE8e-nCas9 expression cassette, $spec^R$，Cm^R	This study
pAX-ABE7.10-nCas9-CDA	pAX-CDA-nCas9 derivative, P_{xylA}-ABE7.10-nCas9-CDA expression cassette, $spec^R$，Cm^R	This study
pJOE8999	*E. coli-B. subtilis* shuttle vector, temperature sensitive replication origin of $pE194^{ts}$ for *B. subtilis*, Kan^R in *E. coli* and *B. subtilis*	Lab stock
pJOE-B2	pJOE8999 derivative, gRNA targeting *bceB* (B2), Kan^R	This study

pJOE-B3	pJOE8999 derivative, gRNA targeting *bceB* (B3), Kan^R	This study
pJOE-B4	pJOE8999 derivative, gRNA targeting *bceB* (B4), Kan^R	This study

Table S4. The protospacer sequences used in this study

sgRNA Sequences (5'-3')	PAM	Purpose
GCCTCCATTATCTAAAGATG	AGG	Targeting *sigE*
CGCAACAAACAGTTCATTAA	TGG	Targeting *bceB* (B1)
AGCCGCTACATTTTGTTCAG	CGG	Targeting *bceB* (B2)
TCAACATTTGCTTGCAGGAC	AGG	Targeting *bceB* (B3)
ACAGCAAGCTGCATATTGCC	GGG	Targeting *bceB* (B4)
GCCCCCGCTTGTAAACGTGT	AGG	Targeting *bceB* (B5)
CACCCATTTGTTTAAAATAA	AGG	Targeting *bceB* (B6)
AACACCATCACCATAATCAT	AGG	Targeting *bceB* (B7)
ATCAGCCAGAAATATTGCAA	AGG	Targeting *pksA* (*pksA-1*)
CACGCGCACAGGGTCAAGCA	TGG	Targeting *pksA* (*pksA-2*)
CATTCAAAGAATAAAGTCGG	AGG	Targeting *pksB* (*pksB*)
TCATCGCATAAAGAAAGATG	CGG	Targeting *pksC* (*pksC*)
GTTCCCAATACTATCACATG	GGG	Targeting *pksD* (*pksD-1*)
ACTCAATCATAAAAATGGCG	GGG	Targeting *pksD* (*pksD-2*)
ACACAAATATCGTCAGCCAT	CGG	Targeting *pksE* (*pksE-1*)
TCCCGCGGCAGACATTGAGA	AGG	Targeting *pksE* (*pksE-2*)
CACGGGATTAGCATATACGG	TGG	Targeting *pksF* (*pksF*)
GCACCGGACAATGATGCGTA	AGG	Targeting *pksG* (*pksG*)
AAACACACAGCTTAAAAAGT	AGG	Targeting *psdB* (P1)
CGCTCAAGCATGTTTTGACA	AGG	Targeting *psdB* (P2)
TAACAGGCCTGCTCCAATGC	CGG	Targeting *psdB* (P3)
CTGAAACAGGATGAGAAAGC	CGG	Targeting *psdB* (P4)
ATTAACACTAAAATCAAAAT	TGG	Targeting *psdB* (P5)

Table S5. The mutations of PsdB

Mutants	Position of mutation
M1	PsdB (V26T/L59P/L126P/I164T, F166L/V236A, L237S)
M2	PsdB (V26A/S60N/L126P/I164T, L165P, F166L/V236A)
M3	PsdB (V26A/L59S, S60N/L126P/L165P, F166L/L235S, V236A)

M4	PsdB (C25R, V26A/L59P/L126P/L165P, F166L/V236A, L237S)
M5	PsdB (V26A/L59S, S60N/L126P/I164T, L165P, F166S/V236A, L237P)
M6	PsdB (V26A/L59S, S60N, A61T/L126P, L127S/F166L/V236A, L237S)
M7	PsdB (V26A)
M8	PsdB (V26A/L235S)

Supplementary Figure

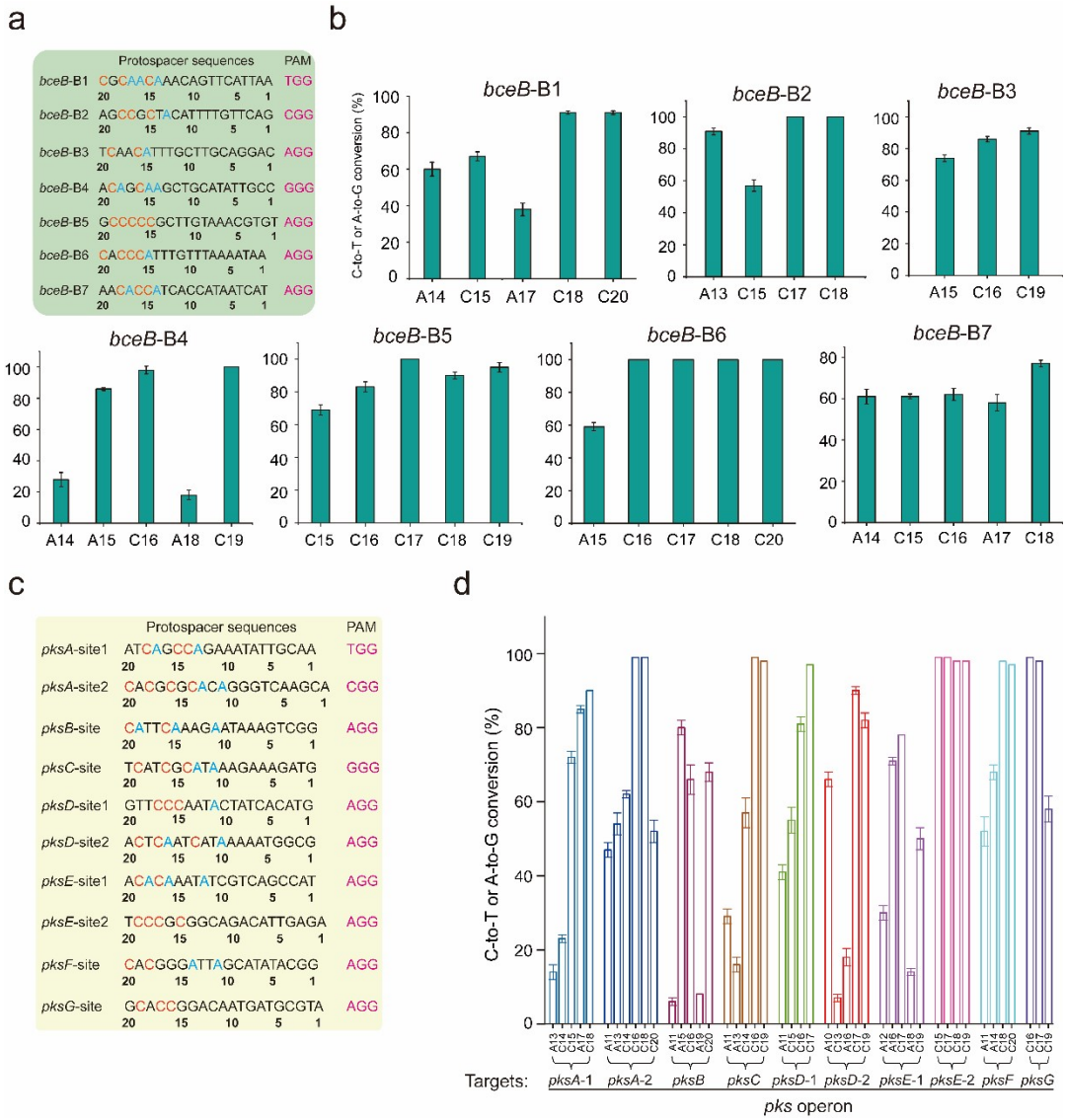

Fig. S1 The base editing efficiency of the dual-base editor for the *bceB* gene and *pks* operon. (a) The protospacer sequences for the editing of *bceB*. **(b)** Base conversion efficiencies of the dual-base editor with the corresponding single sgRNA (B1-B7) targeting *bceB*. **(c)** The protospacer sequences for the editing of *pks* operon. **(d)** Base conversion efficiencies of the dual-base editor with the corresponding single sgRNA (*pksA-1*, *pksA-2*, *pksB*, *pksC*, *pksD-1*, *pksD-2*, *pksE-1*, *pksE-2*, *pksF*, and *pksG*) targeting *pks* operon.

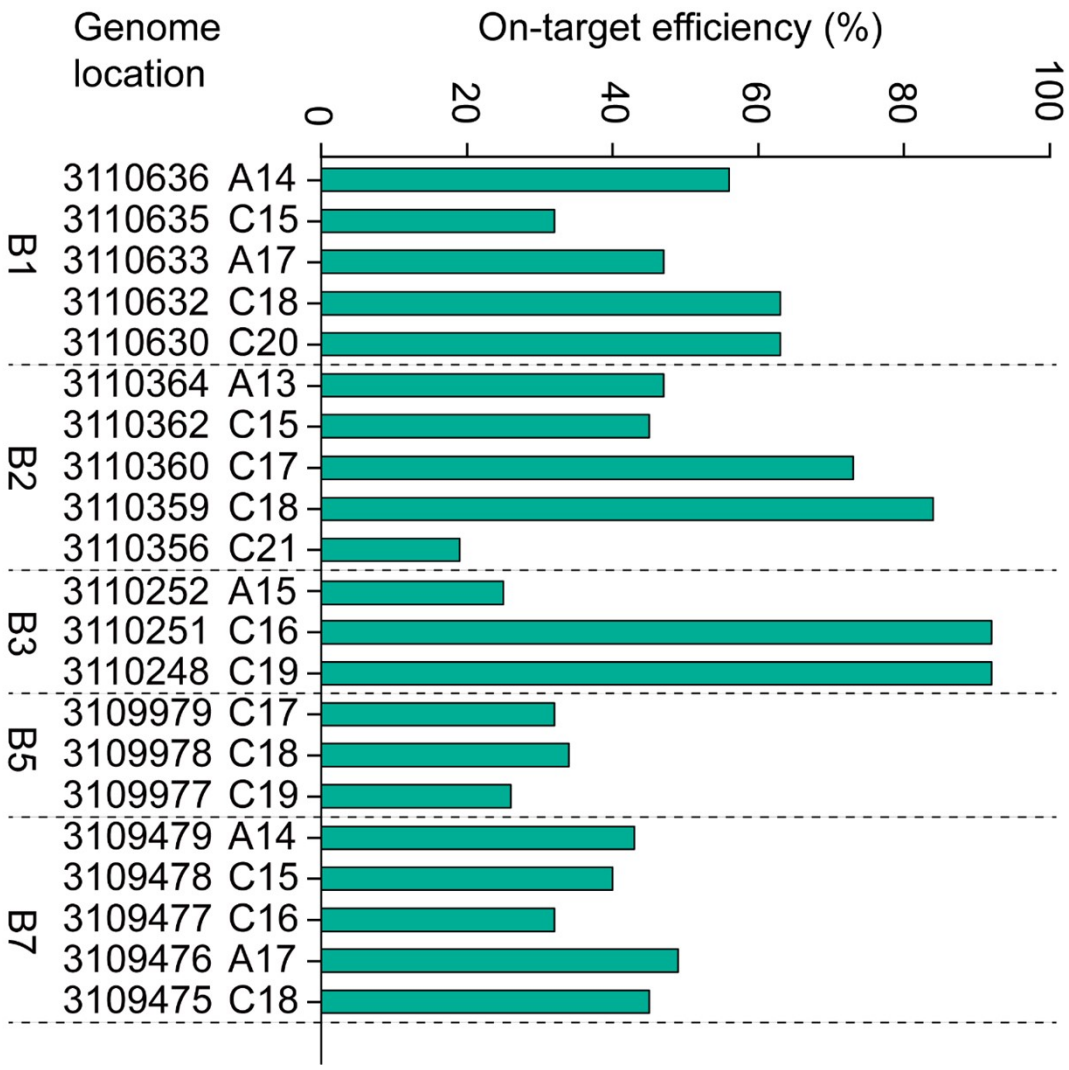

Fig. S2 Genome-wide off-target evaluation of dual-base editor. The high-throughput sequencing results shown that there were no mutations at non-specific sites except for the sgRNA-targeting specific sites (B1, B2, B3, B5, and B7). Thus, the contrast on-target efficiencies of *bceB* sites (B1, B2, B3, B5, and B7) were shown. The corresponding positions of DNA on-target are indicated at the genome-scale. Corresponding sgRNA sequences targeting *bceB* are listed in Table S4. The high-throughput sequencing data of this study are available at the Sequence Read Archive (PRJNA808834) of the NCBI.

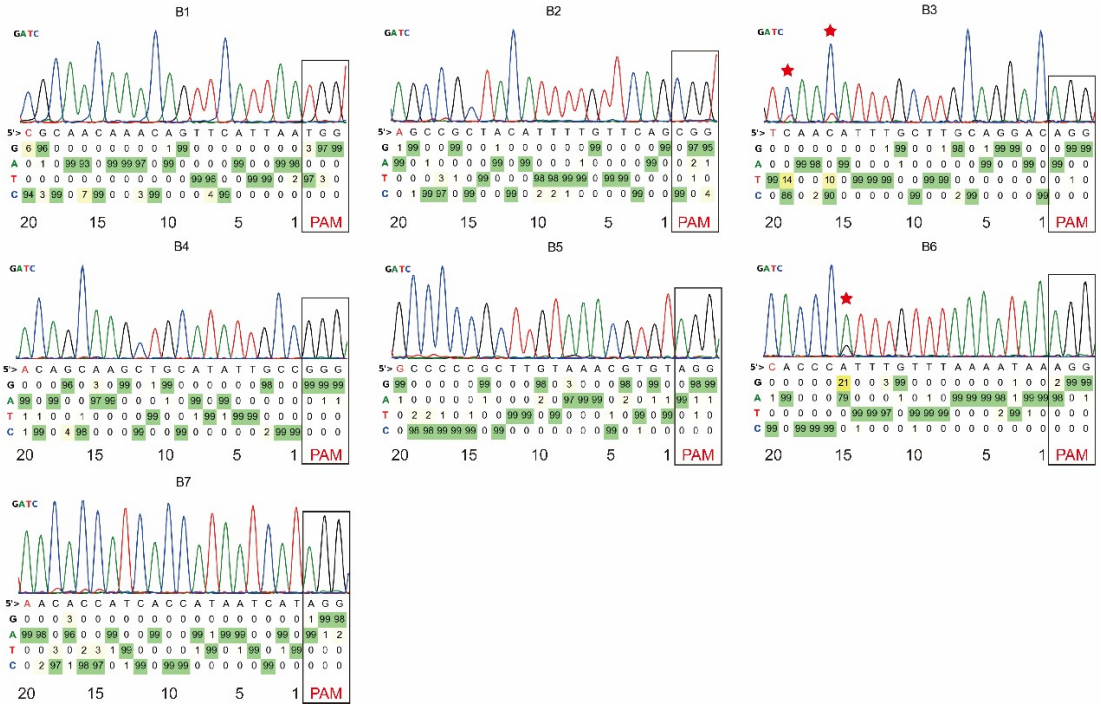

Fig. S3 Sample image output of the sequencing data of the editing of *bceB* from the BEAT analysis. The base conversion efficiencies of the dual-base editor with an sgRNA array (B1-7) targeting *bceB* under the absence of xylose. The raw sequencing data is quantitatively analyzed by BEAT[2]. The order of partial bases is numbered and PAM motifs are framed with black rectangle. The mutated bases are indicated in red stars.

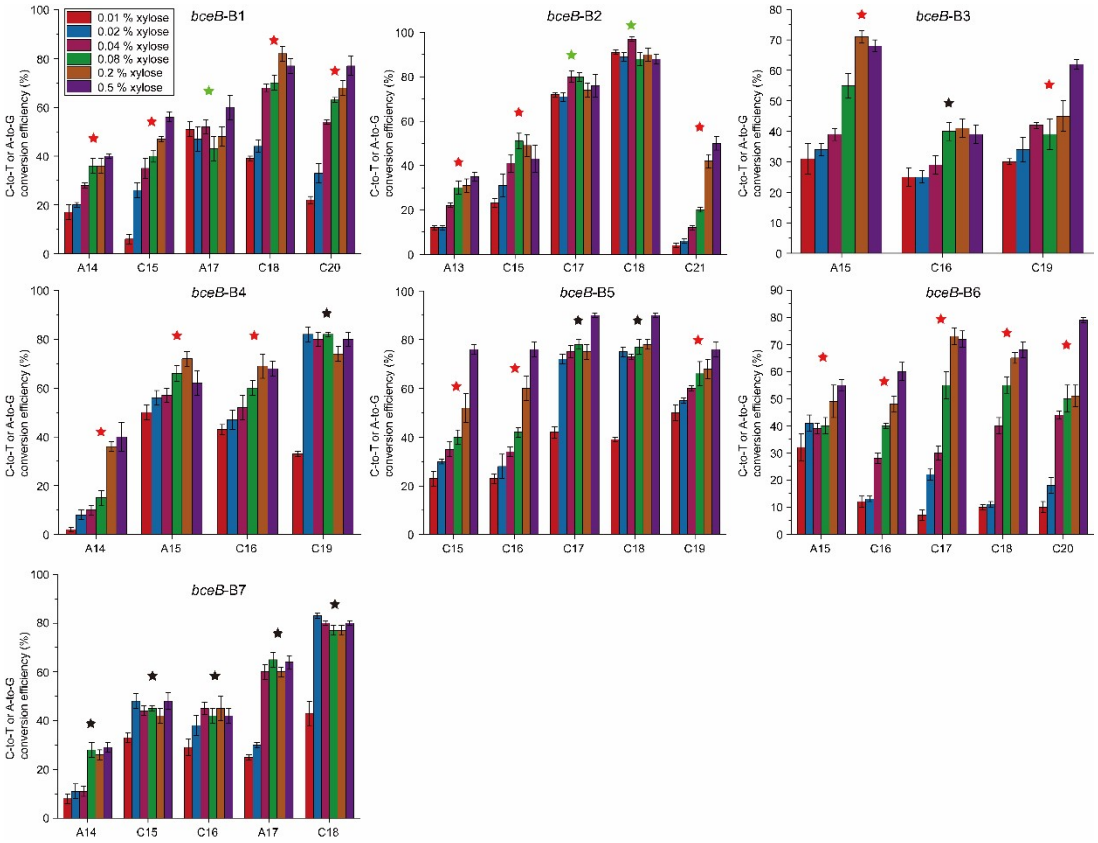

Fig. S4 Base conversion efficiencies of the dual-base editor with an sgRNA array (B1-7) targeting *bceB* at different xylose concentration. Quantitative analyses of base conversion efficiencies of the dual-base editor for the editing of *bceB* with an sgRNA array (B1-7). Similar editing pattern is indicated with stars of different colors.

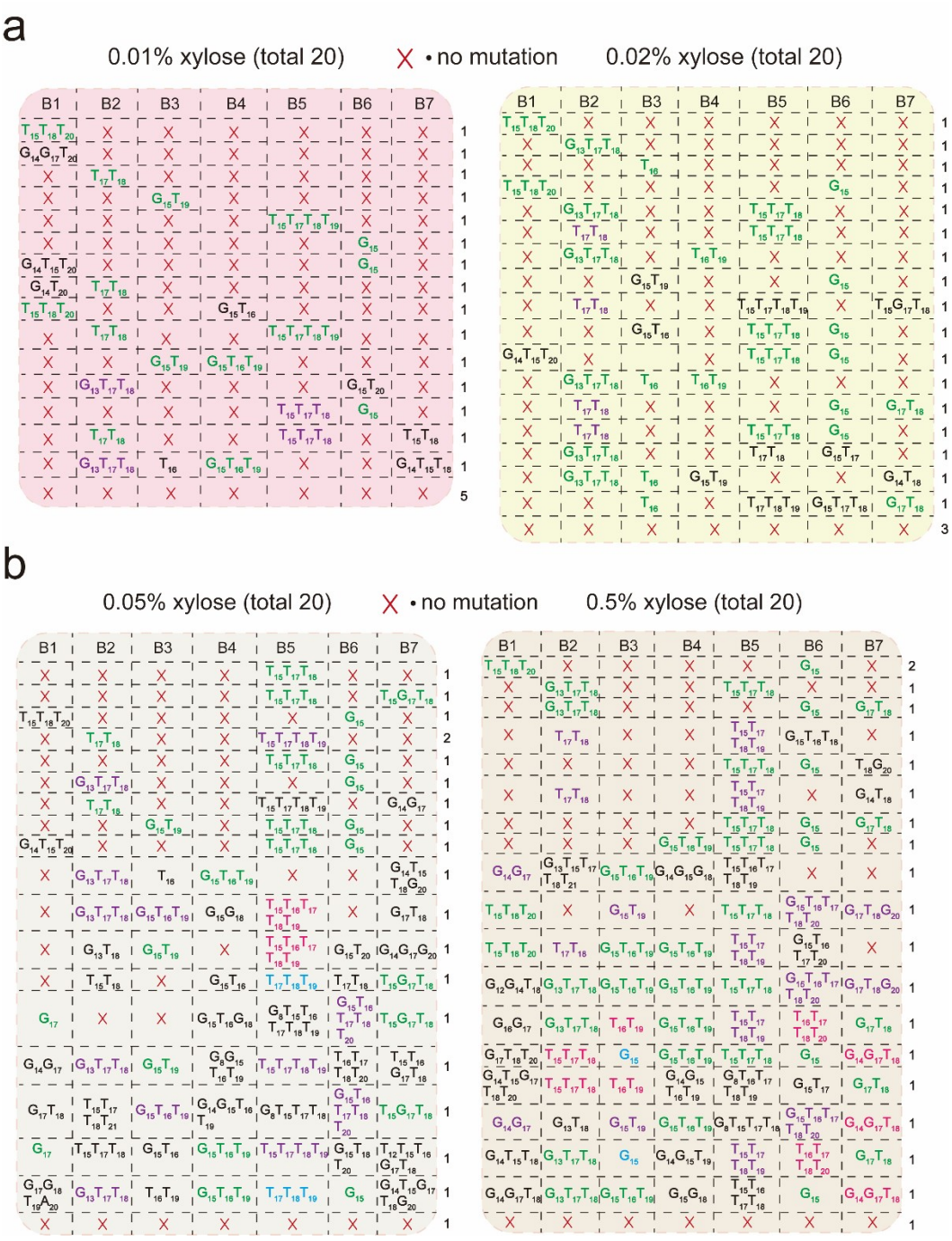

Fig. S5 Identification of genetic diversity for multiplex editing of *bceB* at the single-clone level under different xylose concentrations. (a) Identification of genetic diversity for multiplex editing of *bceB* with an sgRNA array (B1-7) under 0.01% and 0.02% xylose concentrations, respectively. **(b)** Identification of genetic diversity for multiplex editing of *bceB* with an sgRNA array (B1-7) under 0.05% and 0.5% xylose concentrations, respectively. The identical genotype at the same site is showed in the same color.

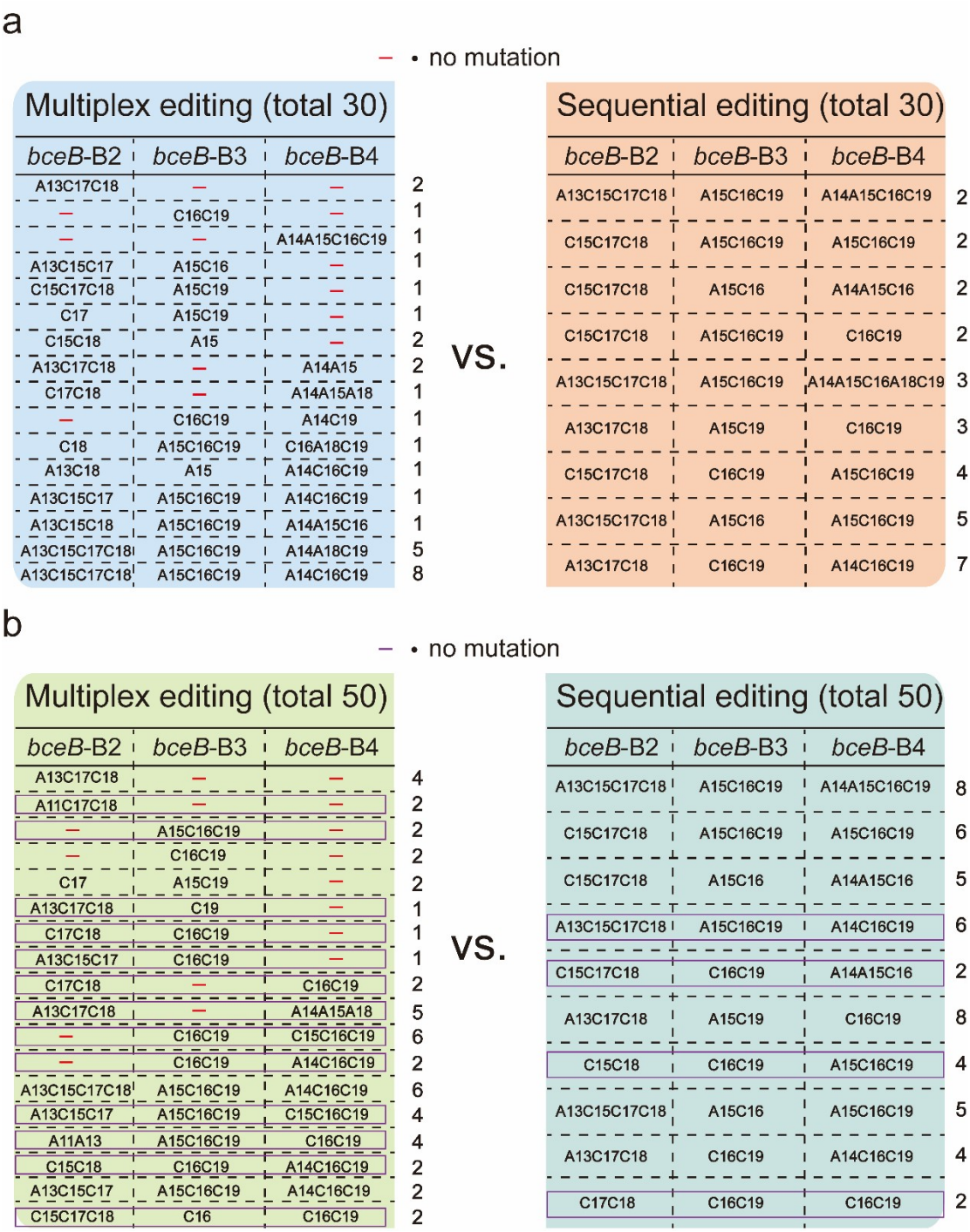

Fig. S6 Identification and comparison of genetic diversification between multiplex editing and multiple rounds of single editing at the single-clone level. (a) Identification of genetic diversification of 30 colonies from multiplex editing system and multiple rounds of single editing system, respectively. **(b)** Identification of genetic diversification of 50 colonies from multiplex editing system and multiple rounds of single editing system, respectively. The purple rectangles suggest that new mutants are different from the previous identification in 30 colonies.

sgRNA / Variants	P1	P2	P3	P4	P5
M1	C15A16C17	A14A15	A16C17	A10A15C20	A14A17
M2	A14A16	C16C18	A16C17	A10A13A15C20	A14C15
M3	A14A16C17	A15C18	A16C17	A13A15C20	A11A14
M4	A12A14A16	A14A15	A16C17	A13A15C20	A14A17
M5	A16C17	A15C18	A16C17	A10A13A16	A14A16A17
M6	A14A16	A15C18C20	A16A19	A15A17C20	A14A17
M7	A16C17				
M8	A16C17				A11

Fig. S7 Identification of PsdB mutants by single-clone sequencing. Six mutants (M1, M2, M3, M4, M5, and M6) with different tolerance to nisin were selected to identify the types of mutations by sequencing. Moreover, the mutants PsdB-V26A (M7) and PsdB-V26A/L235S (M8) were also identified by sequencing. P1-5 denote sgRNAs targeting different loci of *psdB*, respectively. All sgRNA sequences targeting PsdB and amino acid substitutions in PsdB are shown in Table S4 and Table S5.

Supplementary method

The preparation of relevant medias:
1) Every 2mL SPI contains 980 μL SPI-A, 980 μL SPI-B, 20 μL Glucose (50%), and 20 μL CAYE;
2) Every 2mL SPII contains 1960 μL SPI, 20 μL $CaCl_2$ (5 mM), and 20 μL $MgCl_2$ (250mM);
3) SPI-A contains 4 g/L $(NH_4)_2SO_4$, 28 g/L $K_2HPO_4 \cdot 3H2O$, 12 g/L KH_2PO_4, and 2 g/L sodium citrate;
4) SPI-B was 0.4 g/L $Mg_2SO_4 \cdot 7H_2O$;
5) CAYE contains 20 g/L casamino acid and 100 g/L yeast extract;
6) SPI-A, SPI-B, 50% Glucose, CAYE, 5 mM $CaCl_2$, 250mM $MgCl_2$, and 10 mM EGTA (adjusting pH to 8.0 by NaOH) were sterilized at 121 °C for 15 min separately.

Supplementary sequences

pHY plasmid related key genetic parts

TATTTTTTTGCCAAAGCTGTAATGGCTGAAAATTCTTACATTTATTTTACAT

TTTTAGAAATGGGCGTGAAAAAAAGCGCGCGATTATGTAAAATATAAANN
NNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATA
AGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT

Annotations:
P43 promoter, alternative 20 base target site, tracr RNA terminator

pDGT plasmids related key genetic parts

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Annotations:
amyE upstream homologous arm, rrnB T1 terminator, rrnB T2 terminator, P43 promoter, RBS, *GFP* gene, lambda t0 terminator, spectinomycin resistant gene expression cassette, *amyE* downstream homologous arm

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Annotations:
amyE upstream homologous arm, rrnB T1 terminator, rrnB T2 terminator, P43 promoter, RBS, *GFP* gene, lambda t0 terminator, Pveg promoter, sgRNA scaffold, spectinomycin resistant gene expression cassette, *amyE* downstream homologous arm
The highlighted part in turn indicates the targeted sequence of B2, B3, B4, B6, B1, B5, and B7.

pAX plasmids related key genetic parts

pAX-ABE7.10-CDA-nCas9
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Annotations:
lacA upstream homologous arm, chloramphenicol resistance gene, lambda t0 terminator, Bacillus megaterium xylR, xylose promoter (Pxyl), RBS, ABE7.10 gene (The highlighted parts indicate linkers), ABE8e gene, PmCDA gene, XTEN linker, GSAASR linker, nCas9 (D10A) gene, rrnB T1 terminator, *lacA* downstream homologous arm

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