Applied Microbiology and Biotechnology

Genome engineering of the *Corynebacterium glutamicum* chromosome by the Dual-In/Out strategy

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SUPPLEMENTARY INFORMATION

Table S1 Primers, used in the current study

Primer	Sequence (5' – 3')	Application
P1	tcgcgtttcattgattaacgac	mini-Mu(LER) unit integration point determination (MuL)
P2	aaccgggaggacattggatt	
P3	taatacatctgtttcatttgaagcg	mini-Mu(LER) unit integration point determination (MuR)
P4	ctgctttttattcattacatggg	
P5	tgtggaagatccaggattagcc	Confirmation of the pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$ plasmid and its derivatives integration into native *attB*$_{\phi16}$
P6	cttggcctgcaacgatgtaa	
P7	gggcaaatcaccgctagtga	
P8	cgggtgagattagtggacgt	
P9	gcgcgcagctgcagatgctgg	Construction of a linear ds-fragment **ds 542Sm** (13869)
P10	cccacagttcgactatttaacg-tcaggaagtgcacgcagctcaa	
P11	ttgagctgcgtgcacttcctga-cgttaaatagtcgaactgtggg	
P12	acaccctcaggttccgcgacc-ctcgcaagctatccccac	
P13	gtggggatagcttgcgag*gg*-tcgcggaacctgagggtgt	
P14	tctcgtcgcggtcgtaaagca	
P15	catatgcgtacatccattgcc	Test primers for confirmation of $RecE^{564}T$ mediated recombination with **ds 542Sm**
P16	cgtgcccaacgtgcgggtgta	
P17	acccaacacctaggtgtcgga	Construction of a linear ds-fragment **ds 1741Sm** (13869)
P18	cccacagttcgactatttaacg-agttgcacgcaactcatccag	
P19	ctggatgagttgcgtgcaact-cgttaaatagtcgaactgtggg	
P20	tgccactgtgactggtttgtc-ctcgcaagctatccccac	
P21	gtggggatagcttgcgag*g*-acaaaccagtcacagtggca	
P22	gagggaatcatcgtggactgc	
P23	gcagaacaagaactcctccct	Test primers for confirmation of $RecE^{564}T$ mediated recombination with **ds 1741Sm**
P24	ggaaggtgacgccgaagactt	
P25	acacatcctgctcgacgaacc	Construction of a linear ds-fragment **ds 1865Sm** (13869)
P26	cccacagttcgactatttaacg*g*-acggcatcaggggagccctc	
P27	gagggctcccctgatgccgtc-cgttaaatagtcgaactgtggg	
P28	cgcgcagacaacaccgacgtc-ctcgcaagctatccccac	
P29	gtggggatagcttgcgag*g*-acgtcggtgttgtctgcgcg	
P30	cgcgcagacaacaccgacgtc	
P31	tgaacgccaacgcgtcctcat	Test primers for confirmation of $RecE^{564}T$ mediated recombination with **ds 1865Sm**
P32	gtggatccatccaacgacgca	
Test primers for confirmation of **mini-Mu(LER)** unit localization at the appropriate point of *C. glutamicum* ATCC13869		
P33	gaccttcctgatcaacattgg	**258** point
P34	tgatggcgtggcagtatatag	
P35	cattatgcgaaagctcactgt	**1883** point
P36	tggacttcatggcagcaatag	

Primer	Sequence (5’ – 3’)	Application
P37	ctcaaaactagcgaaggatct	**2123** point
P38	tacgagtggaagcaaacggaa	
P39	gcagcatatggattgtggca	**35** point
P40	ttgttaaaagccaccatggt	
P41	ttggaaatgcgatcacggtag	**198** point
P42	cacaaagtttacgaggacaga	
P43	tttcgacgtactaaagaacg	**209** point
P44	agatctcttcgaagttcaca	
P45	acgaggactgcataaactgc	**400** point
P46	gactagcccaggcgtttatc	
P47	tgaaagagtcaggtccatac	**668** point
P48	cctaaccaacactcgtgaaa	
P49	agatgacttggtgacagatgtt	**1213** point
P50	tttacgtggctcttcaactt	
P51	atggccagtgggaagctttgaa	**1275** point
P52	aaggagaaggacggacgcga	
Test primers for confirmation of **mini-Mu(LER)** unit localization at the appropriate point of *C. glutamicum* ATCC13032		
P53	gatcaacgaacacaccgatttcac	**177** point
P54	cagtgcaggaatggtgactc	
P55	atcatcagtgcttgttccagaa	**544** point
P56	tgaaaaatccaacactccag	
P57	ggagactgtcacgatttcca	**657** point
P58	tacagttggaagcctgaccc	
P59	atgtcaatgctcccgtactg	**2020** point
P60	gttcagtcgttgcgagatcc	
P61	cattggtgggaattgcttac	**2393** point
P62	ggattgtgacggtgtcgaca	
P63	gtaaccatcactctcaccatgct	**3244** point
P64	caagtgatcagatttcctctgt	
Test primers for confirmation of **mini-Mu(LER)** unit localization at the appropriate point of MB001		
P65	ggtttcggtagcacggttta	**190** point
P66	ctggtccgggaatgctaata	
P67	tatctgggtggtggagttatga	**837** point
P68	cctcactagtacgcggataa	
P69	accatgcgtaagggtattca	**1128** point
P70	ctgaatctgatgcgcgaaac	
P71	ccagtcaacgtcattaatca	**1320** point
P72	ctgcctagcgattcctaaag	
P73	tggcaggtaggacgtacaatta	**1540** point
P74	cctatgcctgaaagtttcct	
P75	ctacgttggtttcgaagtgc	**2684** point
P76	tcgaatgatggaatacacac	
P77	agatcgtgcagcggcgtactc	Test primers for **mini-Mu(LER)** unit (in tandem with test primers
P78	cccagatcatatgaaacaaca	

Primer	Sequence (5’ – 3’)	Application
		on the appropriate point of chromosomes)
P79	aaccaaggaggagtccacaag	Construction of a linear ds-fragment **ds 400Cm** (13869); Test primers for confirmation of RecE^{564}T mediated recombination P45/P46
P80	cccacagttcgactatttaacg-cagggctaaaactcttatcct	
P81	aggataagagttttagccctg-cgttaaatagtcgaactgtggg	
P82	ggaccgaacaccccgtctttg-ctcgcaagctatccccac	
P83	gtggggatagcttgcgag-caaagacggggtgttcggtcc	
P84	gaggaattcatcggtgatgat	
P85	attccgctgaggtttgattttg	Construction of a linear ds-fragment **ds 668Cm** (13869); Test primers for confirmation of RecE^{564}T mediated recombination P47/P48
P86	cccacagttcgactatttaacg-ccgaagcaatcgcactggccaa	
P87	ttggccagtgcgattgcttcgg-cgttaaatagtcgaactgtggg	
P88	tacgacgacacagaaggtgttt-ctcgcaagctatccccac	
P89	gtggggatagcttgcgag-aaacaccttctgtgtcgtcgta	
P90	gaatccattgtggacaaagatc	
P91	ctttctcgcctagcgcaattg	Construction of a linear ds-fragment **ds 2370Cm** (13869)
P92	cccacagttcgactatttaacg-ctgctgtttttcctcatcgct	
P93	agcgatgaggaaaaacagcag-cgttaaatagtcgaactgtggg	
P94	gtgctcatgtgcgcgcctttt-ctcgcaagctatccccac	
P95	gtggggatagcttgcgag-aaaaggcgcgcacatgagcac	
P96	tttcctactcatgcggttcgt	
P97	aggagacacccccgatataca	Test primers for confirmation of RecE^{564}T mediated recombination with **ds 2370Cm**
P98	tcgggtcaccaagtcagatac	
P99	tatataacgcgt-agtgccaagcttgcatgcctg	Primers for cloning P_{dapA}-*yEGFP* fragment into pCRIM-Cm^{R}-*lox*-*attP*$_{\phi16}$; MluI and XhoI underlined
P100	ttaacacctcctgtcgacagc	
P101	gctgtcgacaggaggtgttaa-atgggtaggagggcttttgta	
P102	atatctcgag- ttacaacactcccttcgtgct	
P103	atatacgcgtggaaatattaaaccttgcatcagg	Primers for cloning P_{cskA}-*turboRFP* fragment into pCRIM-Cm^{R}-*lox*-*attP*$_{\phi16}$
P104	tctgttccctcctttctagattcctttgccggtttatttgt	
P105	tctagaaaggagggaacagaatgagcgagctgatcaaggag	
P106	atatctcgagttatctgtgccccagtttgc	
P107	tgagatgatcgaggtagtcat	Construction of a linear ds-fragment **ds 2393Sm** (13032)
P108	gtggggatagcttgcgag-cgctgcagtcaggatcttctt	
P109	aagaagatcctgactgcagcg-ctcgcaagctatccccac	
P110	ggttgataaataaggtctggatttc-cgttaaatagtcgaactgtggg	
P111	cccacagttcgactatttaacg-gaaatccagaccttatttatcaacc	
P112	ggattcacacccgtagtagtt	
P113	cgttcaaactcattatccaga	Test primers for confirmation of RecE^{564}T mediated recombination with **ds 2393Sm**
P114	gtcaggcagttctgcgaatag	
P115	atacgctgccagctcatgaattc-tgtcaaacatgagaattacaacttatatcg	Construction of pSTV-Cm^{R}-(Int-*attP*)$_{\phi16}$
P116	ggagtagagacgaaagtgcacgagctc-tgatttagtgtatgatggtgtttttgagg	
P117	gtgtggattttgtcggatcagcttgagtaggacaaatccgccgagcttcgacgag-attttcaggagctaaggaagctaaa	
P118	cttcccaacagttgcggg-cgtttaagggcaccaataactg	
P119	gaattcatgagctggcagcgtatttgaccgatccggacacctgggataat-gtgtggattttgtcggatcag	

Primer	Sequence (5’ – 3’)	Application
P120	ctaaatcagagctc-gtgcactttcgtctctactcc	
P121	tgcccttaaacgccc-gcaactgttgggaagggcgat	
P122	ccatttacgatcagctggtca	
P123	cggccgctaaatcggccaaat	
P124	ccgatcgcccttcccaacagt	
P125	catatgggcagactacctact	
P126	atacttacacgcacctttcg	
P127	catatgggcagactacctact	
P128	taccgttcgtataatgtatgctatacgaagttat-gagctctgatttagtgtatga	Construction of pCRIM-Cm^R -*lox-attP*$_{\phi16}$
P129	ataacttcgtatagcatacattatacgaacggta-gtgcactttcgtctctactcc	
P130	ataacttcgtataatgtatgctatacgaacggta-gcggccgctaaatcggccaaa	
P131	taccgttcgtatagcatacattatacgaagttat-cgatcgcccttcccaacagtt	
P132	tatata-gaattcatgagctggcagcgtatt	
P133	tatata-cccggg- cgtttaagggcaccaataactg	Construction of pUCIDT-Ap^R-L-Cm^R-R pUCIDT-Ap^R-L-Sm^R-R
P134	tcaccataatgaaataagatctctac	
P135	cggaacgcgcttggctgtgag	
P136	cgggtatata-gggtaccgagctcgaattcac	
P137	tatatatatatctagacataggca-ccgttaacgtcaacaaccacc	
P138	tatgtctagatatatatatataggatc-ccatttacgatcagctggtcag	
P139	atgggtcgacctgcag-ttagctaagcctgtgacctgg	
P140	ctcggtaccc-tatata- cccggg- acggtaactgatgccgtatttg	
P141	tgtctcataacccaattctct-ggatcc-gggaccgagctcgaattacgt	
P142	acgtaattcgagctcggtccc-ggatcc-agagaattgggttatgagaca	Construction of pVC-Am^R-$Int_{\phi16}$-SceI
P143	ctaactgcaggtcgacccatgg-actatcgctaatcgtgatcgc	
P144	tatataatcgatggaaggagctgactgggttga	
P145	gttaattaacctcctatcgat-gctagctctagatcctgtgtg	
P146	atcgataggaggttaattaac-atgcatcaaaaaaaccaggtaat	
P147	ttatttcaggaaagtttcggag	
P148	tgtctcataacccaattctctg	
P149	attaccctgttatcccta-gatcgaaatgaccacagg	
P150	ggatcccctatata-ggatcc-atctgagactttactttg	
P151	gtacgttttcgactcactcatatg-gtgtctcctctaaagattgta	
P152	tacaatctttagaggagacaccat-atgagtgagtcgaaaacgtac	
P153	ggtaagtggtgcagtttaggcggccgc-ccttatatttttgattcatgc	
P154	gcatgaatcaaaaatataagggcggccgc-ctaaactgcaccacttacc	Construction of pVC-Km^R-(Xis/Int)$_{\phi16}$
P155	caaaaccccgcatcaccaaag	
P156	tctagacataggcaccgttaa	
P157	ggatcccatttacgatcagct	
P158	ctacggaaggagctgtggacc	
P159	tatata-gtcgac-agtcgattggctgagctcatg	
P160	gataagtcgaccgatcgcccttcccaacag	
P161	tgagggagccacggttgatga	
P162	cgggtatata-gggtaccgagctcgaattcac	
P163	tccggaagatctcataggca-ccgttaacgtcaacaaccacc	
P164	ctcggtaccc-tatata-cccggg-acggtaactgatgccgtatttg	
P165	tgtctcataacccaattctct-ggatcc-gggaccgagctcgaattacgt	Construction of pVC-Am^R-LacI-$P_{trc\text{-}id2}$-$RecE^{564}T$
P166	acgtaattcgagctcggtccc-ggatcc-agagaattgggttatgagaca	
P167	gtaatgc-ccatgg- actatcgctaatcgtgatcgc	
P168	cgatagt-ccatgg-gcattacaccgccaggctgaa	

Primer	Sequence (5’ – 3’)	Application
P169	taatctgagttgaggttaaaaaacaatg-gaacatccgcacaatgagaatgct	
P170	tttaacctcaactcagatta-gctagctctagatcctgtgtg	
P171	tgcctatgagatcttccgga -atcgat- ggaaggagctgactgggttga	
P172	tgtggaagatccaggattagcc	Construction of pBS5TΔB
P173	ctcgcaagctatccccacaag-aacccacagttcgactaattaacg	
P174	cgttaattagtcgaactgtgggtt-cttgtggggatagcttgcgag	
P175	ccaagggttagagcctcatc	
P176	ccaatgagcatgaggatcgt	
P177	tgacaacggtggaggaacta	
P178	gacggcggctttgttgaataa	
P179	ctgttccaaaggctttctag-ccatggtcgtcacagagctgg	Construction of p06-Km^R-P_{dapA}-Cre
P180	ctttacgcgttaaaaaggac-ctagcggagtgtatactggct	
P181	gtccttttaacgcgtaaag-ccacgttgtgtctcaaaatc	
P182	ctagaaagcctttggaacag-ggcggagcgctgaaataagtg	

The sequences homologous to the template are separated by a dash

Recombinant plasmids construction

1. pSTV-Cm^R-(Int-*attP*)$_{\phi16}$

To construct the plasmid pSTV-Cm^R-(Int-*attP*)$_{\phi16}$ (3823 bp) an intermediate construction pSTV-Cm^R was obtained by Gibson Assembly (NEB, USA) from three PCR-mediated amplicons: the first fragment (1099 bp) containing *E. coli* replicon p15A, was amplified with pSTV28 plasmid (TaKaRa) (Table 1) as a template using primer pair P115/P116; the second fragment P_{GA1}-*cat* (841 bp) containing gene *cat* of Tn9 transposon (resistance to Cm) was amplified with pSTV28 plasmid as the template using primer pair P117/P118 to form 791 bp liner fragment followed by its fusion with a strong phage promoter P_{GA1} (Pátek et al. 1996) by PCR using primer pair P118/P119; the third fragment (274 bp), containing the T_{674}-MCS- T_{L3} cassette (a multiple cloning site surrounded by the terminators T_{674} of the corynephage *ϕ*674 and T_{L3} of the phage λ) was amplified with a synthesized cassette [T_{674}-MCS-T_{L3}] (Integrated DNA Technologies) using primers P120/P121. Then P_{native}-*int*-*attP*$_{\phi16}$ fragment (1697 bp), containing *ϕ*16 phage integrase gene *int* with its own promoter, and the *attP*$_{\phi16}$ site located downstream along the *ϕ*16 genome, was amplified from the *ϕ*16 DNA by the primer pair P122/P123, processed by kinase and ligated with a PCR-fragment (2126 bp), amplified with intermediate construction pSTV-Cm^R as a template using primers P124/P125, resulting in the final plasmid pSTV-Cm^R-(Int-*attP*)$_{\phi16}$.

2. pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$

To construct the integrative plasmid pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$ (2609 bp) (GenBank OK651220) PCR-fragment (2541 bp) amplified using the plasmid pSTV-Cm^R-(Int-*attP*)$_{\phi16}$ as a template and primer pair P126/P127, was processed by kinase and circled resulting in intermediate construction pSTV-Cm^R-*attP*$_{\phi16}$, not containing the full-size *ϕ*16*int* gene. To introduce two mutant *lox* – sites (Albert et al. 1995) two fragments (1972 bp) and (705 bp) were amplified from the intermediate construction pSTV-Cm^R-*attP*$_{\phi16}$ using primers P128/P131 and P129/P130, respectively. Amplicons fusion provided according to Gibson Assembly resulted in the final plasmid pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$.

3. pUCIDT-Ap^R- L-Cm^R-R and pUCIDT-Ap^R- L-Sm^R-R

The plasmids pUCIDT-Ap^R-L-Cm^R-R (GenBank OK651225) and pUCIDT-Ap^R- L-Sm^R-R (GenBank OK651226) were used as templates for amplification of the central part of the linear fragments. The plasmids were constructed on the basis of the plasmid pUCIDT-Ap^R-L-MCS-R (3437 bp) (Table 1) containing a synthesizing cassette [*attL*$_{\phi16}$-T_{674}-MCS-T_{L3}-*attR*$_{\phi16}$] (Integrated DNA Technologies) cloned into the vector pUCIDT-Ap^R (2752 bp). To construct pUCIDT-Ap^R-L-Cm^R-R plasmid (4278 bp) P_{GA1}-*cat* fragment (841 bp) was obtained by PCR using the plasmid pSTV-Cm^R-*lox*-*attP*$_{\phi16}$ as a template and pair primers P132/P133, processed by kinase and cloned into the *Hpa*I site of the pUCIDT-Ap^R-L-MCS-R plasmid. To construct pUCIDT-Ap^R-L-Sm^R-R plasmid (4639 bp) P_{serC}-*aadA2* fragment (1202 bp), containing gene *aadA2* (resistance to Sm from pCG4 plasmid (NC_004945.1)) under the control of *C.glutamicum serC* gene promoter, was amplified with p07sR (AGRI collection) as a template by primers P134/P135, processed by kinase and cloned into the *Hpa*I site of the pUCIDT-Ap^R-L-MCS-R plasmid.

4. pVC-Am^R-Int$_{\phi16}$-SceI

Initially, the intermediate plasmid pVC-Am^R-Int$_{\phi16}$ (7171 bp) was obtained by Gibson Assembly of three fragments. The first fragment (4189 bp), containing the *E.coli* pMB1 and *C. glutamicum* pBL1 replicon was amplified from the pVC7N (LC425431.1) plasmid as a template and primer pair P136/P137. The second fragment P$_{native}$-*int* (1484 bp), containing the *ϕ*16*int* gene under the transcription control of its native promoter was amplified by PCR from *ϕ*16 DNA using primers P138/P139. The third fragment (1558 bp) containing the fusion of the *aac(3)*-IV gene (resistance to Am) under the control of its native promoter with terminator T$_{674}$ was obtained by OE-PCR using primer pair P140/P143 and two PCR fragments (1299 bp and 307 bp) amplified with plasmid pPK103 (AGRI collection) and *ϕ*674 phage DNA as the templates by primer pairs P140/P141 and P142/P143, respectively.

To construct pVC-Am^R-Int$_{\phi16}$-SceI (9585 bp) (GenBank OK651222), LacI-P$_{trc–id2}$-I-SceI cassette (2396 bp, containing the DNA fragment with I-*Sce*I meganuclease gene under the control of P$_{trc}$-id2 (P$_{trc}$/O$_{lac}$-id-O$_{lac}$) promoter fused with a divergently transcribed *lacI* gene), was obtained by OE-PCR using primers P144/P147), and two PCR fragments (723 bp and 1694 bp) amplified with the plasmid-templates pUC19RP12 (AF170481.1) and pVC-Am^R-LacI-P$_{trc}$-id2-*yeCitrine* (AGRI collection), primer pairs P146/P147and P144/P145, respectively, followed by processing by kinase and cloning into the *Sma*I site of the plasmid pVC-Am^R-Int$_{\phi16.}$ I-*Sce*I restriction site was introduced into the resulting construction by PCR using divergent primers P148/P149 (the sequence of primer P149 contained I-*Sce*I site). Resulting fragment (9585 bp) was processed by kinase and circled by ligase to form the final plasmid pVC-Am^R-Int$_{\phi16}$-SceI.

5. pVC-Km^R-(Xis/Int)$_{\phi16}$

To construct pVC-Km^R-(Xis/Int)$_{\phi16}$ (8107 bp) (GenBank OK651223), P$_{gap}$-*ϕ*16*xis*-T$_{nus}$ cassette (700 bp) was introduced into the intermediate plasmid pVC-Am^R-Int$_{\phi16}$. The cassette, consisted of the excisionase gene *xis* of *ϕ*16 phage under the control of the *C. glutamicum* promoter P$_{gapA}$ and terminator T$_{nus}$, was obtained by OE-PCR of three fragments. The fragments were amplified using the appropriate templates and primers: P$_{gapA}$ (*C. glutamicum* ATCC13869 chromosome P150/P151); *xis* (*ϕ*16 phage DNA, P152/P153); T$_{nus}$ (*C. glutamicum* ATCC13869 chromosome, P154/P155). Fragments P$_{gapA}$ (451 bp) and *ϕ*16*xis* (246 bp) were overlapped using primers P150/P153, the resulting fragment P$_{gapA}$-*ϕ*16*xis* (652 bp) was further fused with T$_{nus}$ (96 bp) using P150/P155 primers. Resulting cassette P$_{gapA}$-*ϕ*16*xis*-T$_{nus}$ processed by kinase was ligated with 7159 bp fragment amplified with pVC-Am^R-Int$_{\phi16}$ template using primer pair P156/P157 to form an intermediate structure pVC-Am^R-(Xis/Int)$_{\phi16}$. To replace the Am^R by Km^R gene of Tn903 transposon, a 7206 bp fragment was amplified with pVC-Am^R-(Xis/Int)$_{\phi16}$ template using primer pair P158/P159, digested by *Sma*I endonuclease and ligated with processed by kinase PCR fragment (1202 bp) containing the Km^R gene with its own promoter, amplified with pVK9 (Nakamura et al. 2012) as a template using primer pair P160/P161 to form a helper plasmid pVC-Km^R-(Xis/Int)$_{\phi16}$. The Km^R gene orientation was selected in accordance with the direction of the original Am^R gene.

6. pVC-Am^{R}-LacI-P$_{trc\text{-id2}}$-RecE^{564}T

The helper pVC-Am^{R}-LacI-P$_{trc\text{-id2}}$-RecE^{564}T (9145 bp) (GenBank OK651221) was obtained by Gibson Assembly of four fragments. The first fragment (4195 bp) containing *E. coli* and *C. glutamicum* replicons (pMB1 and pBL1, respectively) was amplified with pVC7N plasmid as a template using primers P162/P163. The second fragment (1549 bp), containing the Am^{R} gene (*aac*(3)-IV) expressed from its native promoter fused with the phage terminator of T$_{674}$, was obtained by OE-PCR using primers P164/P167 and two PCR-fragments (1299 bp and 298 bp), amplified with pPK103 plasmid and ϕ674 phage DNA as a templates, using primers P164/P165 and P166/P167, respectively. The third fragment (1776 bp), containing a shortened gene (915 bp) of recE^{564} protein and a full-size gene *recT* of *E. coli* Rac prophage, was amplified from the MG1655 chromosome using primer pair P168/P169. The fourth fragment (1705 bp) containing a promoter P$_{trc\text{-id2}}$ and a divergently transcribed gene *lacI*, was obtained by PCR using pVC-Am^{R}-LacI-P$_{trc\text{-id2}}$-*yeCitrine* (AGRI collection) as a template and primer pair P170/P171.

7. pBS5T-ΔB

To construct plasmid pBS5T-ΔB (9350 bp) two PCR-fragments (944 bp and 940 bp) were amplified with *C. glutamicum* ATCC13869 chromosome as a template using primer pairs P172/P173 and P174/P175, respectively and fused by OE-PCR using primers P176/P177 to form a 1706 bp fragment. The resulting fragment was processed by kinase and cloned into *Sma*I site of the plasmid pBS5T. The orientation of cloned fragment was confirmed using P177/P178 primers, the resulting plasmid pBS5T-ΔB was used to construct the deletion of the "functional core" of the *attB*$_{\phi 16}$ site.

8. p06-Km^{R}-P$_{dapA}$-Cre

Plasmid p06-Km^{R}-P$_{dapA}$-Cre (6111 bp) (GenBank OK651224) was obtained by Gibson Assembly of two fragments. The first fragment (5019 bp), containing *E. coli* replicon p15A, gene of Cre recombinase of phage P1 under the control of the *C. glutamicum* promoter P$_{dapA}$ and a shortened (unstable) replicon pCG1 (Tauch et al. 2003), was obtained by PCR using p06-PdapA-*cre* as a template (MG014197, Gorshkova et al. 2018) and primer pair P179/P180. The second fragment (1132 bp) containing the Km^{R} gene of the transposon Tn903 with its native promoter was amplified with a pVK9-derived plasmid using primers P181/P182.

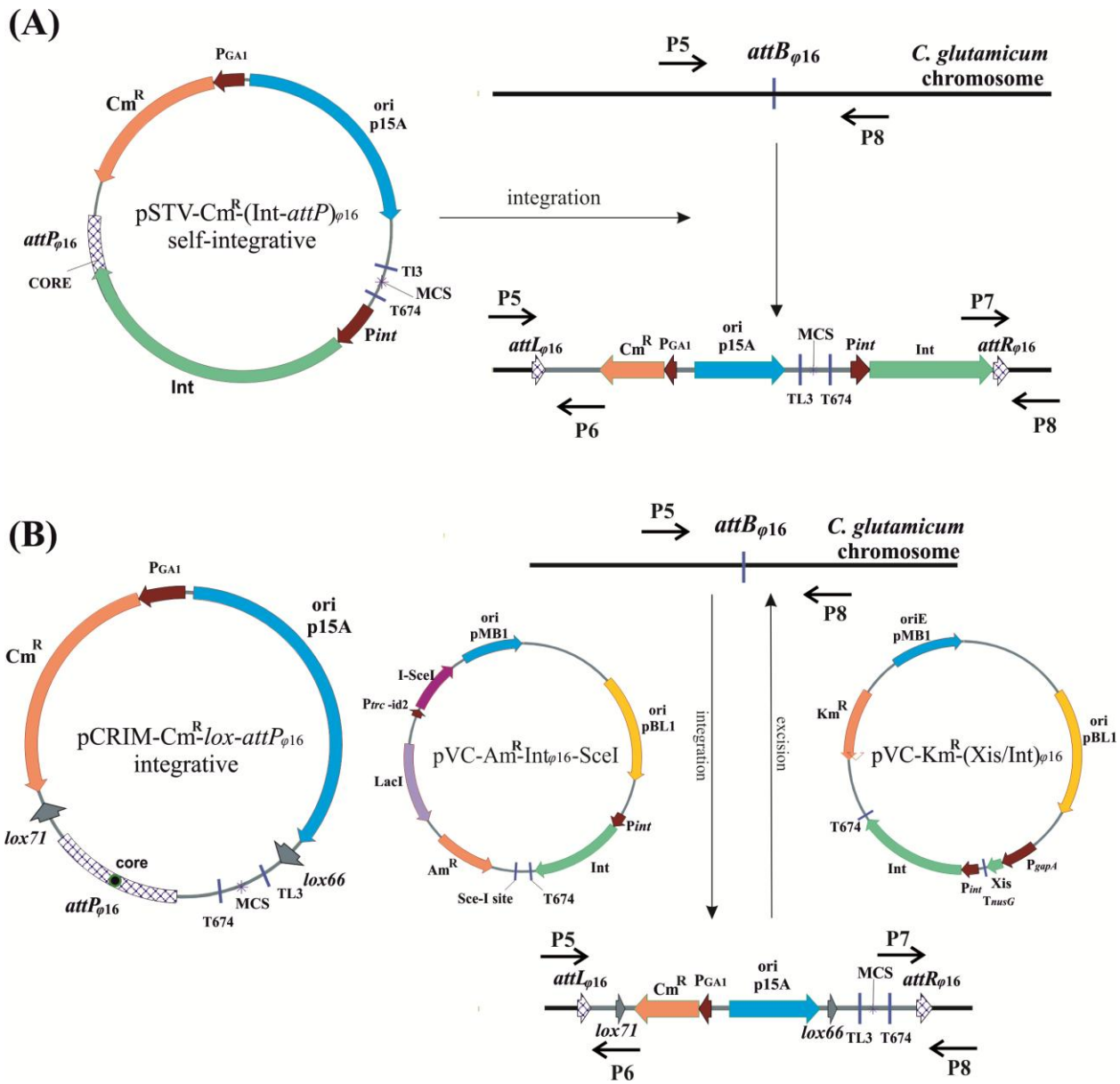

Fig. S1 Schematic representation of "*in cis*" and "*in trans*" *ϕ*16-mediated SSR

(**A**) Integration "*in cis*" of pSTV-Cm^R-(Int-*attP*)$_{\phi16}$ self-integrative plasmid into native *attB*$_{\phi16}$ site on *C. glutamicum* chromosome. Integration occurs due to *ϕ*16 integrase-mediated recombination within *attP*$_{\phi16}$ site (located on the plasmid) and unique *attB*$_{\phi16}$ of *C. glutamicum*. (**B**) Integration/exsicion "*in trans*" of pCRIM-Cm^R-*lox-attP*$_{\phi16}$ (OK651220) integrative plasmid into/out of the native *attB*$_{\phi16}$ on *C. glutamicum* chromosome. Phage *ϕ*16 integrase from the helper plasmid pVC-Am^R-Int$_{\phi16}$-SceI (OK651222) mediates the "*in trans*" insertion of pCRIM-Cm^R-*lox-attP*$_{\phi16}$ integrative plasmid, carrying *attP*$_{\phi16}$ that resulted in incorporation of a circular plasmid into genome. Excision helper plasmid pVC-Km^R-(Xis/Int)$_{\phi16}$ (OK651223), expressing both *ϕ*16 integrase and excisionase genes mediates an excision of pCRIM-Cm^R-*lox-attP*$_{\phi16}$ from *C. glutamicum* chromosome resulting in reconstruction of native *attB*$_{\phi16}$ site. Phage *ϕ*16 integrase and excisionase genes are marked in green, promoters in brown, antibiotic resistance genes in orange, *lox*-sites in grey, *E. coli* and *C. glutamicum* origins of replication in blue and yellow, correspondingly. Phage *attP*$_{\phi16}$ and recombinant *attL/R*-sites are criss-cross hatched. Terminators are indicated with vertical bars, multiple cloning site (MCS) with asterisk, primers are marked by arrows

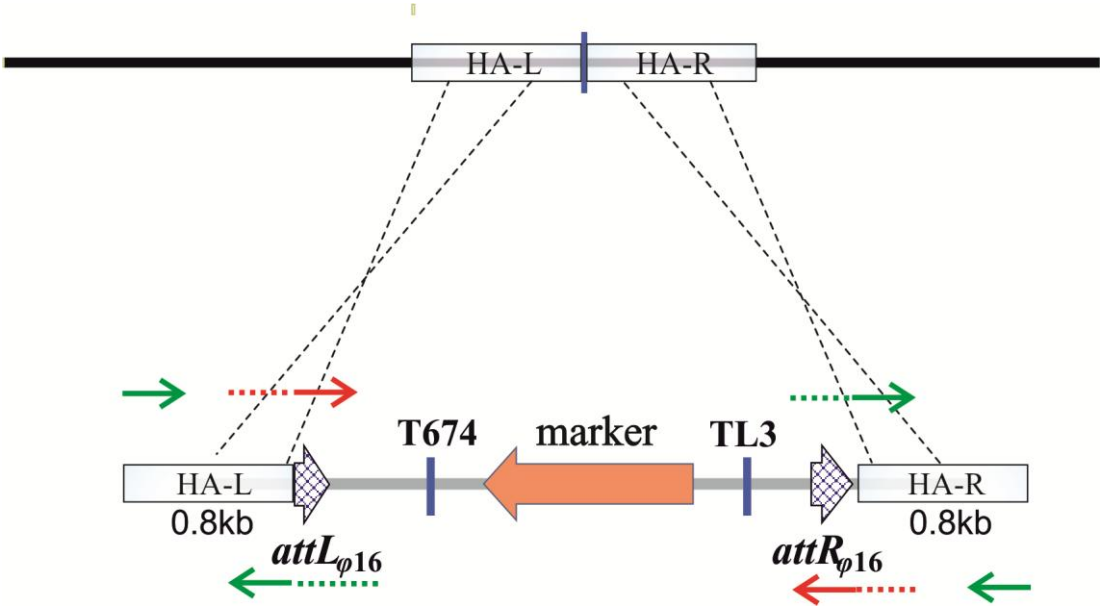

Fig. S2 Scheme of linear dsDNA fragment design for RecE^{564}T mediated recombineering

All linear fragments used in the study were designed in such a way to integrate marker "into a point" without loss of nucleotide sequence. Linear fragment consisted of the antibiotic resistance marker (orange) surrounded by terminators T_{L3}, T_{674} (vertical bars) and flanked by phage $(attL/R)_{\phi16}$ recombinant sites (criss-cross hatched) and two 0.8 kb length arms homologous to target chromosome (rectangles). The central part [$attL_{\phi16}$-T_{674}-Cm^R/Sm^R-T_{L3}-$attR_{\phi16}$] was amplified using a specially prepared non-replicative in *C. glutamicum* plasmids pUCIDT-Ap^R-L-Cm^R-R, pUCIDT-Ap^R-L-Sm^R-R, respectively, as a templates (OK651225, OK651226). Primers for central part amplification are marked with red arrows, primers for homology arms are indicated as green arrows. The dotted line of primer shows region for overlapping with the adjacent fragment. Three PCR-mediated amplicons were assembled in a single molecule by overlapping PCR

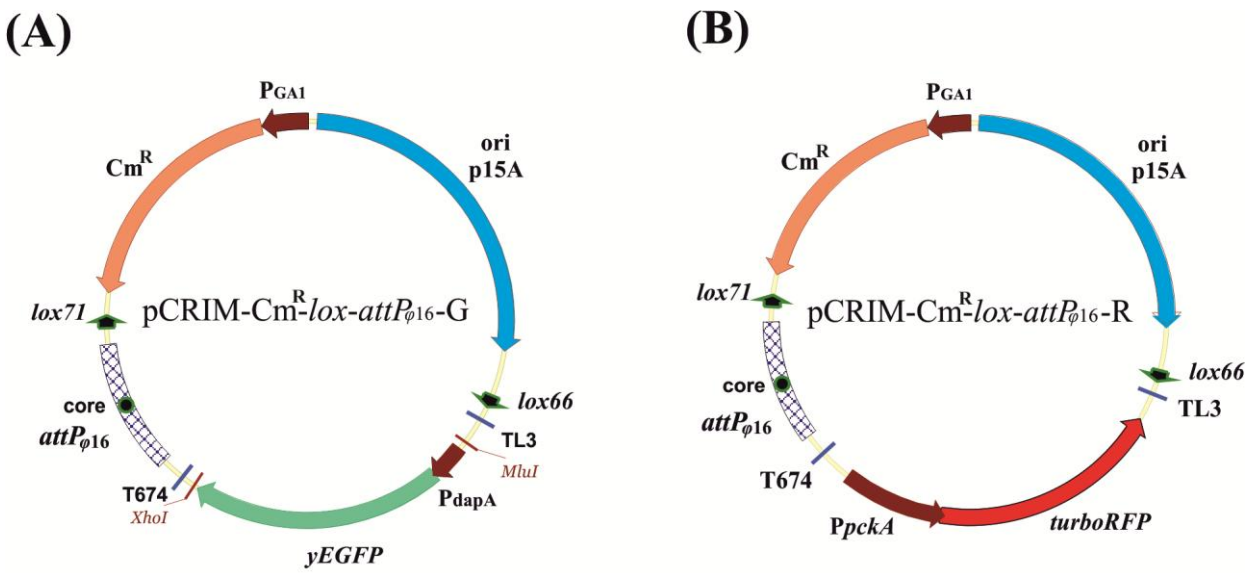

Fig. S3 Integrative plasmids carrying the genes encoding yEGFP and TurboRFP fluorescent proteins

The base integrative plasmid pCRIM-Cm^R-*lox-attP*$_{\phi16}$ (OK651220) is not able to replicate in *C. glutamicum* and contains *attP*$_{\phi16}$ site, MCS for cassette of interest cloning, surrounded by T_{L3}, T_{674} terminators, antibiotic marker and *E. coli* replicon p15A flanked by *lox66/71*- sites for further Cre-mediated excision. On the base of this plasmid two new plasmids were obtained by cloning the gene of fluorescent protein yEGFP under constitutive P_{dapA} (*C. glutamicum*) into *Mlu*I/*Xho*I using primers P108-P111, and TurboRFP gene under P_{cskA} (*E. coli*) into *Eco*RV site using P112-P115, resulting pCRIM-Cm^R-*lox-attP*$_{\phi16}$-G and pCRIM-Cm^R-*lox-attP*$_{\phi16}$-R plasmids, correspondingly