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

Juliya S. Lobanova [a], Natalya V. Gorshkova [a,*], Alexander A. Krylov [a], Nataliya V. Stoynova [a], Sergey V. Mashko [a,b]

[a] *Ajinomoto-Genetika Research Institute, 117545 Moscow, Russian Federation*
[b] *Biological Department, Lomonosov Moscow State University, 119991 Moscow, Russian Federation*

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ABSTRACT

A novel genome editing method for repeated introduction of foreign DNA, including insertion of rather large DNA fragments, into predesigned points in the *Corynebacterium glutamicum* chromosome was developed. The method is based on the implementation of the Dual-In/Out strategy, which was previously provided in *Escherichia coli* according to recombineering-based methods (Minaeva et al., 2008) and allowed step-by-step construction of marker-less plasmid free recombinant strains. The strategy, suggested in the current study, is based on (*i*) *E. coli* Rac prophage RecE^{564}/RecT-dependent recombineering; (*ii*) corynephage ϕ16 (Int/Xis)- and *E. coli* phage P1 Cre-mediated site-specific recombination systems; and (*iii*) the development of a *C. glutamicum* electrotransformation protocol with donor chromosomal DNA for combining of obtained modifications. It was found, that for each tested *C. glutamicum*s strain, the efficiency of the different modifications for electrotransformation fluctuated significantly (up to two orders of magnitude), likely due to the recombinogenic accessibility of the corresponding locus of the bacterial chromosome. To avoid this difficulty, we proposed the phage Mu-driven transposition as a powerful approach for pre-selection of chromosomal regions convenient for single insertions and their further combination in a one strain. Additionally, it was found that the expression of RecE^{564}/RecT coding genes in the recipient strain facilitated the inheritance of the penetrated DNA. It is proposed that the developed strategy in general and its separate elements should be helpful for broadening the genetic toolbox needed for genome editing of targeted *C. glutamicum* strains.

1. Introduction

Corynebacterium glutamicum, a gram-positive soil bacterium, originally isolated because of its natural ability to excrete L-glutamate (Kinoshita et al., 1957), has become an important workhorse for large-scale industrial biotechnology (Becker and Wittmann, 2012). This microorganism is generally recognized as safe and is used in the fermentative production of various substances from biofuels, polymers, bulk and fine chemicals to feed additives and high-value compounds for nutritional and pharmaceutical applications (Baritugo et al., 2018; Becker et al., 2018). Techniques for genome editing of *C. glutamicum* are continuously improving (for review, see Wang et al., 2021).

Early *C. glutamicum*-based producer strains were obtained by repeated rounds of random mutagenesis followed by selection for desired qualities. Later producers were constructed by site-directed mutagenesis mainly based on homologous recombination (HR), which allowed realization of chromosome modifications such as deletions, replacements, and rather short insertions. This method utilizes various integrative plasmids that could not replicate in the corynebacterial host used and only survive by single-cross-based integration into the genome due to the presence of an extended homology region. For scar-less genetic modification, a second single-cross HR-based event must occur at another point of homology to remove the vector backbone and retain the modified region of the bacterial genome (Nešvera and Pátek, 2011; Schäfer et al., 1994; Schwarzer and Pühler, 1991). To facilitate the selection of clones with rare double crossover events resulting in content exchange between the chromosome and the inserted recombinant plasmid, counterselectable markers, such as *sacB* (Tan et al., 2012), *upp* (Ma et al., 2015) or *rpsL* (Kim et al., 2011; Wang et al., 2019), are mostly used in *C. glutamicum*. The efficiency of this approach was significantly improved by using the intron-encoded I-*Sce*I endonuclease gene from *Saccharomyces cerevisiae* (Jacquier and Dujon, 1985; Colleaux et al.,

* Corresponding author.
E-mail address: natalia_gorshkova@agri.ru (N.V. Gorshkova).

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1986) combined with the corresponding I-*Sce*I recognition site to create a double-strand break (DSB) as a unique counterselection tool (Wu et al., 2020). Recently developed DNA-editing strategies based on CRISPR/Cas9 (Cho et al., 2017; Liu et al., 2017) or the CRISPR-Cpf1 (Jiang et al., 2017) system currently encompass a contraselective set of tools that can facilitate marker-less gene deletion, gene insertion, precise base modification, and double-locus editing in *C. glutamicum*.

The efficient recombineering (Copeland et al., 2001; Ellis et al., 2001) method based on the *Escherichia coli* Rac prophage RecE/RecT system was recently adapted for genome engineering in *C. glutamicum* (Huang et al., 2017), as well. This method exploits a phage-encoded HR system consisting of two proteins, 5′→3′ dsDNA-dependent exonuclease RecE and ssDNA annealing protein RecT (Hall et al., 1993), to promote a significantly higher efficiency of recombination than the host-encoded HR system. To date, only a full-length variant of the RecE protein along with RecT has successfully facilitated HR in *C. glutamicum* (Huang et al., 2017; Zhao et al., 2020; Luo et al., 2021; Li et al., 2021), whereas the different truncated variants of RecE that retain the C-terminal domain (260 aa residues in length compared to the 866 aa residues of the full-size RecE) with exonuclease activity (Zhang et al., 2009), e.g., $RecE^{588}$ or $RecE^{602}$ (i.e., C-terminus of RecE starting at residue 588 or 602, respectively), and RecT have been repeatedly used for recombineering in other bacteria (Zhang et al., 1998; Fu et al., 2012). It was shown that in *C. glutamicum*, the RecE/RecT system could catalyze as a minimum "linear plus circular" HR (Huang et al., 2017; Li et al., 2021; Fu et al., 2012), e.g., between a linear dsDNA fragment with a removable selectable marker bracketed by arms of approximately 800 bp homologous to the targeted sequence, and a circular bacterial chromosome. This recombineering could lead to target gene disruption, rather large (up to 40,000 bp) genome deletions, and relatively short DNA insertions. Moreover, the RecE/RecT system was recently successfully combined with Cre/*loxP* (Huang et al., 2017; Luo et al., 2021) or CRISPR/Cpf1 (Zhao et al., 2020) for multiple gene editing and dsDNA-based large marker-less deletions.

However, although HR-based methods could provide any type of chromosomal modifications, it was known that (*i*) the increasing of insertion length between homologous flanking sequences from 1.2 kb strongly decreased recombination efficiency (Huang et al., 2017); (*ii*) it was rather difficult to insert additional long-length DNA fragments already present in the bacterial genome.

Different recombineering-based strategies have already been developed for the integration of large DNA fragments (Rivero-Müller et al., 2007) and second and subsequent copy introduction (Igonina et al., 2020) into the chromosomes of various organisms. Similar tools, based mainly on modified transposons (Suzuki et al., 2006) and, recently, on the phage Mu-derived system (Gorshkova et al., 2018), have already been adjusted for *C. glutamicum*.

The dual-component Mu-transposition system allows integration of the target cassette as a part of a transposing mini-Mu unit from an integrative plasmid into the *C. glutamicum* chromosome, followed by its possible intrachromosomal amplification up to the desired number of copies depending on the expression of genes encoding MuA and MuB transposition factors. The significant disadvantage of this method for targeted genome editing is the possible uncontrolled rearrangements of the bacterial genome (including deletions and inversions of large chromosomal DNA fragments) coupled with intrachromosomal mini-Mu unit amplification (Akhverdyan et al., 2011).

Extended and repeated insertions can also be introduced into the *C. glutamicum* chromosome using the phage site-specific recombination (SSR) system. The size of the inserted DNA fragment is limited only by vector capacity, and the temporarily induced activity of these systems, as a rule, is not toxic to the bacterial host. So-called CRIM (conditional-replication, integration, and modular (Haldimann and Wanner, 2001)) vector plasmids have also been constructed. In the case of *Corynebacteria*, this type of vector could possess the replicon from an *E. coli* plasmid and carry the specific *attP* site of some temperate phages, e.g., corynephages ϕ16 (Moreau et al., 1999a),ϕ304L (Moreau et al., 1999b), the β-phage of *C. diphtheriae* (Oram et al., 2007), and ϕAAU2, which infects *Arthrobacter aureus* C70 (Le Marrec et al., 1996), for possible site-specific integration into the corresponding chromosomal *attB* site via SSR governed by cognate "helper" plasmids. Since wild strains usually have 1–2 native *attB* sites for each temperate phage, additional artificial sites for SSR-based integration can also be introduced into the bacterial genome.

Since each of the genome editing methods has its own advantages and disadvantages, there are a number of studies demonstrating the positive effects of synergistic combinations of different systems and elements in one strategy (Suzuki et al., 2005a, 2005b).

A good example of such synergistic combination is the Dual-In/Out strategy developed for *E. coli* (Minaeva et al., 2008) as a convenient method for introducing extended insertions (with experimentally confirmed insertions of more than 10–15 kb (Haldimann and Wanner, 2001; Igonina et al., 2020)) in a predesigned location of a bacterial chromosome with the possible following consecutive combination of the set of marked mutations in a single strain, e.g., by sequential P1*vir* based general transduction (P1-duction) or electrotransformation by purified chromosomal DNA.

The aim of the current study was to develop the Extended Dual-In/Out strategy for *C. glutamicum* since almost all elements of this process have already been reported separately. To the best of our knowledge, only P1-like transduction and chromosomal electrotransformation as possible methods for combination of individual marked modifications in the strain of interest have not been demonstrated for *C. glutamicum* to date.

Our adjustment of the previously developed Dual-In/Out strategy includes the following: (*i*) construction of *C. glutamicum* strains with a differently located, unique precursor of the artificial $attB_{\phi16}$ site accomplished by targeted $RecE^{564}$/RecT-mediated integration of an antibiotic resistance marker bracketed by hybrid $(attL/R)_{\phi16}$sites, each extended with ~800 bp homologous arms, into the bacterial chromosome (the first **"In"**); (*ii*) conversion of precursors for $attB_{\phi16}$ as a scar in the chromosome after $(Xis/Int)_{\phi16}$-mediated marker excision (the first "**Out**"); (*iii*) cloning of target genes into the appropriate CRIM vector carrying an $attP_{\phi16}$ site, followed by $Int_{\phi16}$-dependent integration of the recombinant CRIM plasmid into the chromosomal $attB_{\phi16}$ site (the second "**In**"); and (*iv*) the possible Cre-derived excision (the second "**Out**") of the vector part, including replication origin and a selectable marker, originally bracketed by *lox66/lox71* sites, out of the chromosome or the postponement of this excision for (*v*) transfer-marked chromosomal modification in another strain that underwent chromosomal DNA electrotransformation. The results of the current study demonstrated that the *C. glutamicum* host-encoded HR system (alone or enhanced in the presence of expressed $RecE^{564}$/RecT proteins) provides double-cross-based HR, resulting in allelic exchange between the bacterial chromosome and large dsDNA homologous fragments, which entered the cell by electroporation.

As a proof of concept, the proposed strategy was successfully applied for gene-engineered editing of the *C. glutamicum* chromosome in two industrially important strains, ATCC 13869 and ATCC 13032.

2. Materials and methods

2.1. Strains, plasmids and cultivation conditions

Information on all of the strains and plasmids used in this study is presented in Table 1.

C. glutamicum strains were grown in BHI liquid medium (37 g L^{-1} brain heart infusion, Sigma–Aldrich, St. Louis, MO, USA) at 30 °C. When needed, the corresponding antibiotics were added at the following final concentrations: kanamycin 25 mg L^{-1} (Km25), chloramphenicol 7.5 mg L^{-1} (Cm7.5), streptomycin 10 mg L^{-1} (Sm10), apramycin 30 mg L^{-1} (Am30) and gentamicin 1 mg L^{-1} (Gm1).

Table 1
Strains and plasmids used in the present study.

Abbreviation in text	Description	Reference or source
C. glutamicum strains		
ATCC 13869	AJ1511 (ATCC 13869 without cryptic plasmid pAM330) Sequence Source: AP017557.2	Laboratory collection
69 *x*::[mini-Mu (LER)]	Sm^R; Series of ATCC 13869 strains with single integrated mini-Mu(LER) Sm^R-unit into *x* point, *x*∈{35, 198, 209, 258, 400, 668, 1213, 1275, 1883, 2123}	Gorshkova et al., 2018; this work
69 B::Cm	Cm^R; ATCC 13869 strain with integrated pCRIM-Cm^R-*lox-attP*$_{\phi16}$ plasmid into the native *attB*$_{\phi16}$ site (2,483,498-2,483,526) i.e., 69 B::[*attL*$_{\phi16}$-*lox71*-Cm^R-p15A- *lox66*-T_{L3}-MCS-T_{674}-*attR*$_{\phi16}$]	This work
69 B::Cm *x*::[mini-Mu (LER)]	Sm^R Cm^R; Derivatives of69 B::Cm strain obtained via chromosomal electroporation with genomic DNA of 69 *x*::[mini-Mu(LER)], Sm^R- strains	This work
69 542::Sm 691741::Sm 691865::Sm	Sm^R; Derivatives of ATCC 13869 strain with integrated dsDNA fragment [*attL*$_{\phi16}$-T_{674}-Sm^R-T_{L3}-*attR*$_{\phi16}$] into 542, 1741, 1865 points, respectively	This work
69 ΔB	ATCC 13869 with scarless deletion of native *attB*$_{\phi16}$ site	This work
69 ΔB 400::Cm 69 ΔB 668::Cm 69 ΔB 2370::Cm	Cm^R; Derivatives of 69 ΔB strain with integrated dsDNA fragment [*attL*$_{\phi16}$-T_{674}-Cm^R-T_{L3}-*attR*$_{\phi16}$] into 400, 668 and 2370 points, respectively	This work
69 ΔB400::B 69 ΔB668::B 69 ΔB2370::B	Derivatives of 69 ΔB 400::Cm, 69 ΔB 668::Cmand 69 ΔB 2370::Cm strains with constructed artificial *attB*$_{\phi16}$ site at 400, 668 and 2370 points, respectively	This work
69 ΔB 400::G-Cm 69 ΔB 2370::G-Cm	Cm^R; Derivative of 69 ΔB 400::B and 69 ΔB 2370::B strains with integrated pCRIM-Cm^R-*lox-attP*$_{\phi16}$-G plasmid into artificial *attB*$_{\phi16}$[*attL*$_{\phi16}$-*lox71*-Cm^R-p15A-*lox66*-T_{L3}-*yEGFP*-T_{674}-*attR*$_{\phi16}$] at 400 and 2370 points, respectively	This work
69 ΔB 668::R-Cm	Cm^R; Derivative of 69 ΔB 668::B strain with integrated pCRIM-Cm^R-*lox-attP*$_{\phi16}$-R plasmid into the artificial *attB*$_{\phi16}$[*attL*$_{\phi16}$-*lox71*-Cm^R-p15A-*lox66*-T_{L3}-*turboRFP*-T_{674}-*attR*$_{\phi16}$] at 668 point	This work
69 ΔB 400::G 69 ΔB 2370::G	Derivative of 69 ΔB 400::G-Cm and 69 ΔB 2370::G-Cm strains obtained due to Cre-mediated excision of vector part,[*attL*$_{\phi16}$-*lox72* -T_{L3}-*yEGFP*-T_{674}-*attR*$_{\phi16}$]	This work
69 ΔB 668::R	Derivative of the 69 ΔB 668::R-Cm strain obtained due to Cre-mediated excision of vector part, [*attL*$_{\phi16}$-*lox72* -T_{L3}- *turboRFP*-T_{674}-*attR*$_{\phi16}$]	This work
69 ΔB 400::G 668::R-Cm	Cm^R; Derivative of 69 ΔB 400::G obtained via chromosomal electroporation with genomic DNA of 69 ΔB 668::R-Cm strain	This work
69 ΔB 400::G 668::R	Derivative of 69 ΔB 400::G 668::R-Cm strain obtained due to Cre-mediated excision of vector part	This work
69 ΔB 400::G 668::R 2370::G-Cm	Cm^R; Derivative of 69 ΔB 400::G 668::R obtained via chromosomal electroporation with genomic DNA of 69 ΔB 2370::G-Cm strain	This work
69 ΔB 400::G 668::R 2370::G	Derivative of 69 ΔB 400::G 668::R 2370::G-Cm strain obtained due to Cre-mediated excision of vector part	This work
ATCC 13032	Wild type, Sequence Source: NC003450.3	VKPM B-41 (Kalinowski et al., 2003)
32 *y*::[mini-Mu (LER)]	Sm^R; Series of ATCC 13032 strains with single integrated mini-Mu(LER), Sm^R-unit into *y* point; *y*∈{177, 544, 657, 2020, 2393, 3244}	This work
32 B::Cm	Cm^R; ATCC 13032 strain with single integrated pCRIM- Cm^R-*lox-attP*$_{\phi16}$ plasmid into the native *attB*$_{\phi16}$ site (2,565,640-2,565,668) i.e., 32 B::[*attL*$_{\phi16}$-*lox71*-Cm^R-p15A- *lox66*-T_{L3}-MCS-T_{674}-*attR*$_{\phi16}$]	This work
32 B::Cm *y*::[mini-Mu (LER)]	Sm^R Cm^R; Derivatives of32 B::Cm strain obtained via chromosomal electroporation with genomic DNA of 32 *y*::[mini-Mu(LER)], Sm^R-strains	This work
32 ΔB	ATCC 13032 with scarless deletion native *attB*$_{\phi16}$ site (2,565,640-2,565,668) according to NC003450.3	This work
32 ΔB 2393::Cm	Cm^R; Derivative of 32 ΔB strain with integrated dsDNA fragment [*attL*$_{\phi16}$-T_{674}-Cm^R-T_{L3}-*attR*$_{\phi16}$] into 2393 point	This work
32 ΔB 2393::B	Derivative of 32 ΔB 2393::Cm strain with constructed artificial *attB*$_{\phi16}$ site at 2393 point	This work
32 ΔB 2393::R-Cm	Cm^R; Derivative of 32 ΔB 2393::B strain with integrated pCRIM-Cm^R-*lox-attP*$_{\phi16}$-R plasmid into the artificial *attB*$_{\phi16}$constructed at 2393 point	This work
32 B::G-Cm	Cm^R; Derivative of ATCC 13032 strain with integrated pCRIM-Cm^R-*lox-attP*$_{\phi16}$-G plasmid into the native *attB*$_{\phi16}$	This work
32 ΔB 2393::R	Derivative of 32 ΔB 2393::R-Cm strain obtained due to Cre-mediated excision of vector part	This work
32 B::G	Derivative of 32 B::G-Cm strain obtained due to Cre-mediated excision of vector part	This work
32 B::G 2393::R-Cm	Cm^R; Derivative of 32 B::G obtained via chromosomal electroporation with genomic DNA of 32 ΔB 2393::R-Cm strain	This work
32 B::G 2393::R	Derivative of 32 B::G 2393::R-Cm strain obtained due to Cre-mediated excision vector part	This work
MB001	ATCC 13032 with in-frame deletion of prophages CGP1, CGP2, CGP3; Sequence Source:CP005959.1	Baumgart et al., 2013
MB001 *z*::[mini-Mu (LER)]	Sm^R; series of MB001 strains with integration of mini-Mu(LER), Sm^R-unit in *z* point, *z*∈{190, 837, 1128, 1320, 1540, 2684}	This work
MB001 B::Cm	Cm^R; MB001 strain with integration of the pCRIM-Cm^R-*lox-attP*$_{\phi16}$ plasmid into the native *attB*$_{\phi16}$ site (2,333,853-2,333,881) i.e., MB001 B::[*attL*$_{\phi16}$-*lox71*-Cm^R-p15A- *lox66*-T_{L3}-MCS-T_{674}-*attR*$_{\phi16}$]	This work
MB001 B::Cm *z*::[mini-Mu (LER)]	Sm^R Cm^R; Derivatives ofMB001 B::Cm strain obtained via chromosomal electroporation with genomic DNA of MB001 *z*::[mini-Mu(LER)], Sm^R-strains	This work
E. coli strains		
TG1	Δ(*lac-proAB*) *supEthi-1 hsd*Δ*5*($r_K^-m_K^-$) [F′ *traD36proAB*+ $lacI^q lacZ\Delta M15$]	VKM IMG-341
LE392	F^- *hsdR514*($r_K^-m_K^-$) *glnV44supF58lacY1* or Δ(*lacIZY*)6 *galK2galT22metB1trpR55*	Laboratory collection

(*continued on next page*)

Table 1 (*continued*)

Abbreviation in text	Description	Reference or source
Plasmids		
pVC-Am^R-LacI-$P_{trc\text{-}id2}$-RecE^{564}T	Am^R; recombination helper plasmid based on *C.glutamicum/E.coli* shuttle vector pVC7N (LC425431.1; Hashiro et al., 2019) carrying medium-copy-number (MCN) *ori*V_{Cg} replicon from pAM330 (Yamaguchi et al., 1986) or pBL1 (Santamaría et al., 1984), high-copy-number (HCN) *ori*V_{Ec} replicon from pMB1 (derivative) or pUC-like (Yanisch-Perron et al., 1985) plasmid pHSG399 (Takeshita et al., 1987)); the resistance gene, Am^R, *apr*^{R} or *aac (3)IV*(X01385) (Paget and Davies, 1996; Yates et al., 2004), was from the pPK103 (laboratory collection) *E. coli* Rac prophage *recE*^{564}*T* genes under IPTG inducible $P_{trc\text{-}id2}$ promoter (Skorokhodova et al., 2006)	This work; GenBank OK651221
pVC-Km^R-(Xis/Int)$_{\phi16}$	Km^R; excision helper plasmid based on vector pVC7N; Km^R gene from Tn903 transposon amplified from pVK9 (Nakamura et al., 2006) phage *ϕ16int* and *ϕ16xis* genes under the control of the native and P_{gapA} promoters, respectively	This work; GenBank OK651223
pVC-Am^R-Int$_{\phi16}$-SceI	Am^R; integration helper plasmid based on vector pVC7N; phage *ϕ16int* gene under its native promoter; I-*Sce*I site; I-*Sce*I encoding gene from the pUC19RP12 plasmid (AF170481.1; Pósfai et al., 1997) under the transcriptional control of inducible promoter $P_{trc\text{-}id2}$	This work; GenBank OK651222
pSTV-Cm^R-(Int-*attP*)$_{\phi16}$	Cm^R; vector for self-integration into the *attB*$_{\phi16}$-site based on pSTV28 vector (TaKaRa),MCN *E. coli* p15A replicon (Selzer et al., 1983); *cat* from Tn9 transposon under control of strong phage promoter P_{GA1}(Pátek et al., 1996); phage *ϕ16int* gene under its native promoter and *attP*$_{\phi16}$; T_{674}-MCS- T_{L3} (a multiple cloning site surrounded by the terminators T_{674} of the *ϕ674* corynephage and T_{L3} of the phage λ)	This work
pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$	Cm^R; integrative vector based on pSTV-Cm^R-(Int-*attP*)$_{\phi16}$, but unlike it does not contain the full-size*ϕ16int* gene; additionally carries *lox66/lox71*(Albert et al., 1995), that flank the vector part	This work; GenBankOK651220
pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$-G	Cm^R; pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$ with (P_{dapA}-*yEGFP*) in MCS, yEGFP encoding gene from pKT128	This work
pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$-R	Cm^R; pCRIM-Cm^R-*lox*-*attP*$_{\phi16}$ with (P_{cskA}-*turboRFP*) in MCS, TurboRFP encoding gene from pTurboRFP-PRL	This work
pUCIDT-Ap^R-L-Cm^R-R	Ap^R; Cm^R; template plasmid based on pUCIDT-Ap^R, *E. coli* pMB1-type replicon; [*attL*$_{\phi16}$-T_{674}-P_{GA1}-*cat*-T_{L3}-*attR*$_{\phi16}$] cassette	This work; GenBank OK651225
pUCIDT-Ap^R-L-Sm^R-R	Ap^R; Sm^R template plasmid based on pUCIDT-Ap^R; *E. coli* pMB1-type replicon; [*attL*$_{\phi16}$-T_{674}-P_{serC}-*aad*A2-T_{L3}-*attR*$_{\phi16}$]; gene *aadA2* (resistance to Sm) from pCG4 plasmid (NC_004945.1)	This work; GenBank OK651226
p06-Km^R-P_{dapA}-Cre	Km^R; Cre-excision helper based on p06-PdapA-*cre* as (MG014197, Gorshkova et al., 2018),*C. glutamicum /E.coli* shuttle (MCN *ori*V_{Cg} truncated variant of pCG1replicon (Ozaki et al., 1984), MCN *ori*V_{Ec} replicon p15A); *cre* recombinase gene under the control of *C. glutamicum* P_{dapA} promoter	This work; GenBankOK651224
pBS5TΔB	Km^R; pBS5T-based (*ori*V^{ts}_{Cg} replicon from pHSC4; *B. subtilis sacB*-gene) (Fukui et al., 2006) plasmid with cloned DNA amplicon for inactivation of native *attB*$_{\phi16}$	This work
pKT128	pFA6a–link–yEGFP–SpHIS5: Ap^R; MCN *ori*V_{Ec} replicon from pBR322 (Balbás et al., 1986), harboring the yEGFP gene	Sheff and Thorn, 2004
pTurboRFP-PRL	Km^R, Neo^R (G418) *E.coli*/Mam shuttle Promoter-probe vector harboring the promoter-less TurboRFP gene, (MCN *ori*V_{Ec} from pUC, *ori*V_{mam} from SV40)	Evrogen cat# FP235
pVK-*lacI*^{Q}-P_{tac}-Mu*AB*	Gm^R; pVK9 (Nakamura et al., 2006)-based vector with cloned the MuA and MuB transposition factors genes under the transcriptional control of IPTG inducible *lacI*^{Q}-P_{tac}	Gorshkova et al., 2018; GenBank MG014199
pAH-mini-Mu (LER)-YS	Sm^R; Km^R; pAH162 (Haldimann and Wanner, 2001) –based vector with cloned transposing DNA in the form of Mu*att*L-T_{his} -[*lox*66-P_{17Mme}-*yECitrine -strAB -lox*71]-T_{deo}-Mu*att*R and genes of Sm^R, Km^R and *sacB*	Gorshkova et al., 2018; GenBank MG014200

E. coli TG1 and LE392 strains were used as cloning hosts for plasmid manipulation and were cultured in Luria-Bertani (LB) medium (10 g L^{-1} peptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl) (Sambrook and Russell, 2001) at 37 °C. When required, the corresponding antibiotics were added to the *E. coli* strain at the following final concentrations: Km50, Cm30, Sm25, Am30, Gm10 and ampicillin 100 mg L^{-1} (Ap100).

2.2. Recombinant DNA techniques

The oligonucleotides used in this work are listed in Table S1.

Restriction, ligation, and electrophoresis were performed according to standard protocols (Sambrook and Russell, 2001). The construction of all recombinant CRIM and autonomously replicating vectors, plasmids and "helpers" is described in detail in the Supplementary material.

Restriction enzymes, T4 DNA ligase, and High Fidelity PCR Enzyme Mix were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Taq DNA polymerase was purchased from Sileks-M (Moscow, Russia), and Gibson Assembly Master Mix was purchased from New England BioLabs (Ipswich, MA, USA). These enzymes were used according to the manufacturers' instructions. DNA sequencing was performed commercially by Evrogen (Moscow, Russia). The DNA synthesis was performed commercially by Integrated DNA Technologies (https://eu.idtdna.com/pages/products/genes-and-gene-fragments/custom gene synthesis). Plasmid DNA was isolated using a Plasmid Miniprep kit (Evrogen). For electrotransformation of chromosomal DNA into *C. glutamicum* strains, genomic DNA was isolated with a Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) followed by essential purification with a phenol–chloroform extraction method (Sambrook and Russell, 2001).

2.3. Electroporation protocol for C. glutamicum

An overnight culture ($OD_{600} \approx 12$) of *C. glutamicum* was added to a test tube containing 5 mL of BHI liquid medium supplemented with 1 mL of 10% glycine and 0.5 mL of 1% Tween 80 up to an $OD_{600} = 0.5$. Cells were grown at 30°C under agitation (250 rpm) to an $OD_{600} \approx 1.5$–2 for approximately 2 h. Next, the cell culture from one test tube was

harvested to make one preparation by centrifugation (0.5 min 13,000 rpm), washed three times in 1 mL sterile water at room temperature, and concentrated in such a way that the total volume, considering the added DNA sample, was equal to 100 μL. These electrocompetent cells were mixed with DNA and immediately transferred to a 0.1-cm sterile, cold electrode chamber for electroporation at 1.6 kV using a MicroPulser™ (Bio–Rad, Hercules, CA, USA). The cells were immediately diluted with 1 mL of BHI medium, and recovery cultivation at 30 °C with agitation was followed by seeding on a 1.6% agar BHI selective media plate and selection of the desired transformants after 1–2 days of growth at 30 °C.

For typical plasmid electrotransformation ≈ 100 ng of plasmid DNA was added. The recovery time of 2 h was the same across all plasmid transformations of all tested *C. glutamicum* strains, except for p06-Km^R-P_{dapA}-Cre plasmid transformation, where the recovery time was equal to 1 h. To achieve high transformation efficiency, cells of the ATCC 13032 strain were exposed to heatshock (46 °C for 6 min) in 1 mL of BHI medium immediately after electroporation.

For recombineering with a PCR-derived linear dsDNA fragment (amplicon), an overnight culture of the *C. glutamicum* strain carrying the helper plasmid pVC-Am^R-LacI-$P_{trc\text{-}id2}$-$RecE^{564}T$ was diluted in BHI + glycine+Tween80 medium as described above and supplemented with Am30 and 2 mM IPTG to induce the expression of $RecE^{564}T$-encoding genes. Two micrograms of amplicon was electroporated into competent cells as a rule. After electroporation, cells were immediately diluted with 1 mL of BHI medium supplemented with 2 mM IPTG and cultivated at 30 °C for 3.5 h with agitation.

The procedure of chromosomal electroporation by 20 μg of purified genomic DNA was the same for all strains, and the recovery time was 2 h.

2.4. Excision of the lox-bracketed vector part

The plasmid p06-Km^R-P_{dapA}-Cre was transformed into the *C. glutamicum* strains for the excision of the *lox66/71*-bracketed and Cm^R-marked vector part from the recombinant CRIM plasmid integrated into the bacterial chromosome. Cells were plated on solid BHI medium supplemented with Km25 after 1 h recovery and incubated at 30 °C for 2 days. The selected Km^R clones were streaked to single colonies on solid BHI medium and cultivated at 30 °C for simultaneous Cre-mediated excision of the Cm^R marker and helper plasmid curing. The resulting colonies were replicated on solid BHI, BHI + Km25 and BHI + Cm7.5 plates to select Cm^S Km^S clones for further confirmation by PCR.

2.5. Plasmid curing (standard technique)

To eliminate any helper plasmids from the corynebacterial strain, a small amount of culture was seeded in a test tube with 5 mL of liquid BHI medium without antibiotics overnight at 30 °C. Then, a 10 μL aliquot of an overnight culture was added to 5 mL of fresh medium and grown overnight again. Then, the second overnight culture was diluted (10^{-5}, 10^{-4}) and seeded onto solid BHI medium plates. The obtained clones were replicated on solid BHI medium supplemented or not with selective antibiotics. As a result, approximately 90% of clones lost the helper plasmid.

2.6. pVC-Am^R-$Int_{\phi16}$-SceI plasmid elimination

To cure the helper plasmid pVC-Am^R-$Int_{\phi16}$-SceI, cultures were grown overnight in liquid BHI medium supplemented with 2 mM IPTG to express the I-SceI meganuclease gene, which recognizes and cleaves its cognate site located in the same plasmid. Next, cells were similarly seeded in dilutions onto plates with solid medium and replicated according to the standard method of plasmid curing described above. More than 50% of clones lost the pVC-Am^R-$Int_{\phi16}$-SceI plasmid.

2.7. Fluorescence intensity assay

First, 0.1 mL aliquots of overnight cultures of tested strains carrying the TurboRFP- and/or yEGFP-encoding genes and control cells without one or both genes were washed with water and diluted 10 times, and 200 μL cellular suspensions of these cells were transferred to black 96-well plates (GBO, Kremsmunster, Austria). Optical density at 600 nm (OD_{600}) and fluorescence intensity (F) were measured using a Tecan Infinity M200 plate reader (Tecan, Austria). The excitation/emission wavelengths for TurboRFP and yEGFP were selected as 540/574 nm and 490/522 nm, respectively. The fluorescence intensity of a blank sample with water was established as the background fluorescence ($F_{background}$). Relative fluorescence intensity (RF) was calculated according to the eq. $RF = [(F_{target} - F_{background})/(OD_{target} - OD_{background})]$ and expressed in arbitrary units.

3. Results

3.1. Chromosomal electroporation-mediated transfer of genetic modifications

Successful application of general transduction (such as P1-duction for *E. coli*, in particular) has not been demonstrated for *C. glutamicum* yet. Thus, for developing an Extended Dual-In/Out strategy, it was crucially important to test the possibility of chromosomal electrotransformation as an alternative way of combining mutations, which has already been described not only for *E. coli* (Kilbane and Bielaga, 1991; Sheng et al., 1995) but also for several other bacteria (Choi et al., 2006; Katashkina et al., 2009). It is known that penetration of DNA into a cell by electroporation does not guarantee the subsequent HR-mediated integration of the introduced dsDNA into the bacterial chromosome, i.e., transformation per se, even in naturally competent bacteria. The integration requires processing of the penetrated DNA, where the whole set of host recombination enzymes must actively and concertedly participate (Lefrançois et al., 1998).

Previously, the set of marked mini-Mu(LER) units was integrated into different points on the *C. glutamicum* chromosome by a developed dual-component Mu-transposition system including integrative vector pAH-mini-Mu(LER)-YS (GenBank MG014200) and helper plasmid pVK-$lacI^Q$-P_{tac}-Mu*AB* (GenBank MG014199) (Table 1; Gorshkova et al., 2018). These mini-Mu units carried Sm^R as a Cre-excisable antibiotic resistance marker.

The positions of 10 insertions randomly distributed along the whole genome of *C. glutamicum* ATCC 13869 were precisely determined according to the previously developed 'inverse-PCR' (Ochman et al., 1988)-like strategy (Zimenkov et al., 2004) and named for simplicity by their approximate coordinates according to a published chromosome map (Table 2). To test the possibility of chromosomal transfer by electroporation, the ATCC 13869-derived mutant strains with mini-Mu insertions in their genomes named **69 *x*::[mini-Mu(LER)]** (*x* represents any corresponding integration point (Table 1)), served as the donors of chromosomal DNA, whereas another ATCC 13869-derived strain with a Cm^R-marker in the native $attB_{\phi16}$-site (2,483,498-2,483,526 position) of the bacterial chromosome, named **69 B::Cm** (Table 1; the construction of the **69 B::Cm** strain can be seen in Item **3.3** of the **Results**),was used as a recipient.

Cm^R Sm^R *C. glutamicum* clones could be easily detected in all independent electrotransformations by purified chromosomal DNA from the donor strains; the efficiency varied for each preparation of DNA from the strains with different mini-Mu integration points (on average from 16 to 676 cfu/20 μg of genomic DNA/10^8 surviving cells (Table 2)) while the electrotroporation of the same recipient **69 B::Cm** by the empty sample not containing the chromosomal DNA resulted in the appearance of only 1–3 Cm^R, Sm^R *C. glutamicum* clones.

The specifically designed PCR-based experiments (list of oligonucleotides are presented in the Supplementary materials (Table S1))

Table 2
Library of marked mini-Mu integration points and efficiency of their transfer by the chromosomal electrotransformation method.

Integration point name	Inserted cassette	Position in genome[a]	Transfer efficiency, clones/ 20 μg DNA/10^8 surviving cells[b,c]
*C. glutamicum*ATCC13869 (GenBank AP017557.2)			
35	mini-Mu(LER),	35,028	415 ± 42
198	Sm^R (3.7 kb)	198,108	16 ± 3
209		209,618	381 ± 64
258		258,090	98 ± 18
400		400,172	564 ± 94
668		668,996	676 ± 102
1213		1,213,827	638 ± 96
1275		1,275,483	487 ± 68
1883		1,883,247	429 ± 62
2123		2,123,379	349 ± 58
*C. glutamicum*ATCC 13032 (GenBank NC003450.3)			
177	mini-Mu(LER),	177,343	210 ± 31
544	Sm^R (3.7 kb)	544,701	7 ± 3
657		657,238	24 ± 4
2020		2,020,649	16 ± 7
2393		2,393,647	120 ± 19
3244		3,244,425	14 ± 5
C. glutamicum MB001 (GenBank CP005959.1)			
190	mini-Mu(LER),	190,010	15 ± 6
837	Sm^R (3.7 kb)	837,920	36 ± 10
1128		1,128,498	121 ± 19
1320		1,320,683	30 ± 6
1540		1,540,151	593 ± 80
2684		2,684,273	470 ± 66

[a] According to the earlier accepted definition of the DNA cassette integration point (Zimenkov et al., 2004), the position of those nucleotides in the known sequence of the host genome is indicated to be directly linked with the first nucleotide from the "right" terminus of the integrated DNA cassette, Mu-*attR*, in particular;

[b] The transfer efficiency is calculated from the results of three experiments.

[c] The empty sample transfer resulted to appearance only 1–3 Cm^R Sm^R *C. glutamicum* clones.

confirmed that the obtained recombinant strains called **69 B::Cm *x*::[mini-Mu(LER)]** carried full-size mini-Mu units in the same chromosomal loci, ***x***, as the corresponding progenitor strains **69 *x*::[mini-Mu (LER)]** that served as the donor in the electrotransformation experiment.

Additionally, in a similar way, the library of Sm^R-marked mini-Mu integrants for widely used *C. glutamicum* ATCC 13032 (GenBank NC003450.3) and MB001 (GenBank CP005959.1) strains indicated **32 *y*::[mini-Mu(LER)]** and **MB001 *z*::[mini-Mu(LER)]**, respectively, were obtained (Table 1). The transfer of each received Mu-derived Sm^R-marked point was performed successfully by chromosomal electrotransformation for *C. glutamicum* Cm^R strains ATCC 13032 and MB001, named **32 B::Cm** and **MB001 B::Cm**, respectively (Table 1; the construction of the **32 B::Cm** and **MB001 B::Cm** strains can be seen in Item **3.3** of the **Results**). The obtained transfer efficiency is shown in Table 2. The correct transfer of the marked units and their proper locations in the same points of the recipient chromosome was verified by PCR analysis with appropriate oligonucleotides (Table S1).

Therefore, the combination of different mutations located at different chromosomal sites on the *C. glutamicum* genome and available for Mu-driven integration was possible. Keeping in mind the usually proposed (Akhverdyan et al., 2011) random distribution of Mu-driven integration points along bacterial chromosomes, the possible transfer of any targeted DNA fragments of *C. glutamicum* DNA by the adjusted electrotransformation procedure was initially expected.

3.2. Design of the Dual-In/Out plasmid set

To use the Dual-In/Out strategy for *C. glutamicum* (Fig. 1 I(A–D)), a set of autonomously and conditionally replicating plasmids was designed and obtained according to standard gene engineering procedures; these were analogous to the constructs developed for the *E. coli* system (Haldimann and Wanner, 2001; Minaeva et al., 2008).

3.2.1. Description of helper plasmid for recombineering

The possibility of providing RecET-mediated recombineering experiments in *C. glutamicum* had been successfully confirmed to date due to efficient HR-based allelic exchange between the targeted chromosomal locus and inserted linear DNA possessing homologous arms with an optimal length of approximately 800–1000 bp (Huang et al., 2017; Luo et al., 2021; Li et al., 2021). Notably, only the full-size Rac prophage *recE* gene encoding a full-sized protein 866 aa residues in length, along with the *recT* gene, was used in all these experiments.

In the present study, the truncated variant of the RecE protein was used for recombineering experiments in *Corynebacteria* for the first time. $RecE^{564}$ was used as it is the most "long" from the earlier tested truncated RecE derivatives (e.g., $RecE^{588}$, $RecE^{595}$, $RecE^{602}$, $RecE^{606}$ (Zhang et al., 1998; Muyrers et al., 2000; Fu et al., 2012) that retains *exo* VIII nuclease activity associated with the exonuclease domain encompassing the last 260 amino acids at the C-terminal of RecE (Chu et al., 1989; Zhang et al., 2009). The decision to use the longest truncated derivative of RecE was mainly based on the desire to eliminate the "heterologous" for *Corynebacteria* part of the Rac prophage *recE* gene with non optimal (according to the %MinMax tool (Rodriguez et al., 2018)) translation of amino acid codons in the coding region of 367–433 a.a., but hopefully retaining the specific recombineering activities typical not only for truncated, but for the full-sized RecE (Fu et al., 2012), as well.

Therefore, the bi-replicon plasmid pVC-Am^R-LacI-$P_{trc\text{-}id2}$-$RecE^{564}$T (GenBank OK651221, Fig. 2A) was constructed to provide recombineering in *C. glutamicum*. The plasmid was designed on the pVC7N shuttle vector (Hashiro et al., 2019) backbone carrying the high-copy-number (HCN) *E. coli* replicon from the pUC-like plasmid pHSG399 and the medium-copy-number (MCN) corynebacterial replicon of the cryptic plasmid pAM330 from *C. glutamicum* ATCC 13869.The plasmidpVC-Am^R-LacI-$P_{trc\text{-}id2}$-$RecE^{564}$T was used for inducible expression of genes encoding the truncated variant of RecE (starting at an additional Met codon and continuing from the Asp^{564} codon until the end of the RecE coding part) and full-size RecT from the *E. coli* Rac prophage. The tightly LacI-regulated hybrid promoter, $P_{trc\text{-}id2}$ = $P_{trc}/O_{lac\text{-}id}$-$O_{lac}$ (with additional "symmetrical" 20-bp $O_{lac\text{-}ideal}$, as in (Lehming et al., 1987; Oehler et al., 1994), located between the "-35" and "-10" of the well-known (Brosius et al., 1985) P_{trc}-O_{lac}-promoter/operator hybrid regulatory region) (Skorokhodova et al., 2006) and *E. coli lacI* gene with its native promoter, that, in combination, provide a highly repressed and IPTG-inducible expression of the controlled genes.

3.2.2. Description of "helper" plasmids based on corynephage ϕ16 Xis/Int-driven SSR system

To date, all essential genetic elements and protein products of the corynephage ϕ16 (Moreau et al., 1995) – Xis/Int-driven SSR system have been characterized and can be efficiently applied for genome engineering in *C. glutamicum*. Temperate bacteriophage ϕ16 could integrate its DNA in the *C. glutamicum* chromosome in the SSR process catalyzed by integrase protein gp33, also known as $Int_{\phi16}$ (Moreau et al., 1999a; Lobanova et al., 2017), probably with the assistance of host factors. This process occurred according to the Campbell model as a double-strand break and reunion of integrated phage and bacterial DNAs precisely between the $attP_{\phi16}$ and $attB_{\phi16}$ sites, respectively, with generation of hybrid $(attL/R)_{\phi16}$ sites that bracketed the linearized prophage DNA inserted into the bacterial chromosome. For excision of phage DNA from the bacterial chromosome during the SSR process, the concerted action of the ϕ16-originating excisionase (gp47 or $Xis_{\phi16}$

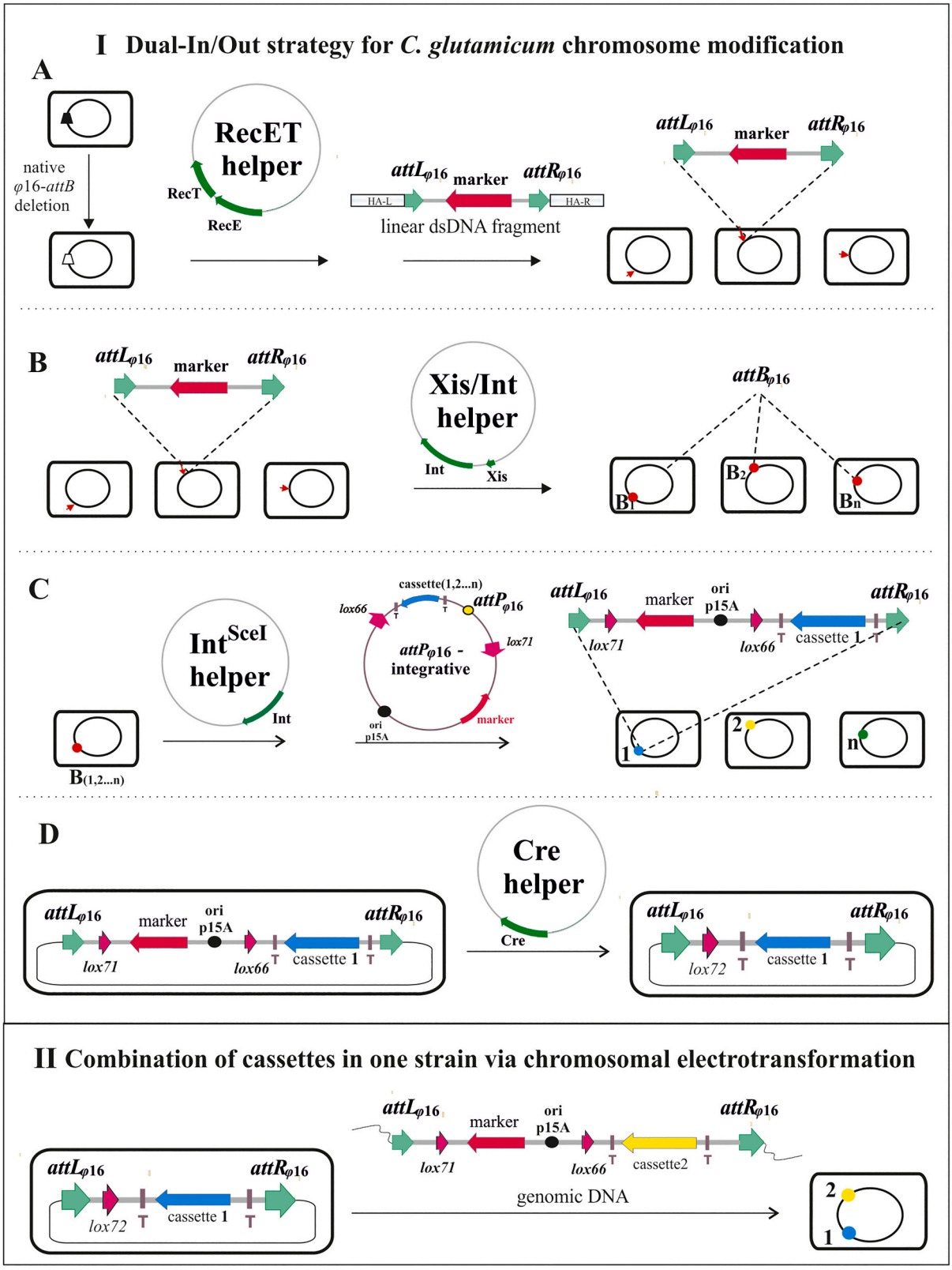

Fig. 1. Schematic representation of the developed chromosome modification method for introduction foreign DNA into a predesigned point on the *C. glutamicum* chromosome. The method is based on the four-step Dual-In/Out strategy (**I A–D**). $RecE^{564}T$-mediated integration of the antibiotic resistance marker bracketed by $(attL/attR)_{\phi16}$ sites into the locus of the *C. glutamicum* chromosome predesigned for further $Int_{\phi16}$-governed integration of the target genes (the first **"In"** in the title of strategy) (A). $(Int/Xis)_{\phi16}$–dependent elimination of the integrated marker (the first **"Out"**) with retention of the artificial $attB_{\phi16}$ –site as the scar in the predesigned locus (B). $Int_{\phi16}$-driven integration (the second – **"In"**) of the recombinant CRIM plasmid with the target cassette and $attP_{\phi16}$ site into the artificial $attB_{\phi16}$ (C). Construction of a "marker-less" recombinant strain due to Cre-mediated excision (the second – **"Out"**) of the vector part of the CRIM plasmid bracketed by *lox66/lox71* with retention of the targeted gene(s) linked with *lox72* as the scar (D). The proposed strategy also implies combining consequently integrated marked cassettes by the chromosome electrotransformation method (**II**).

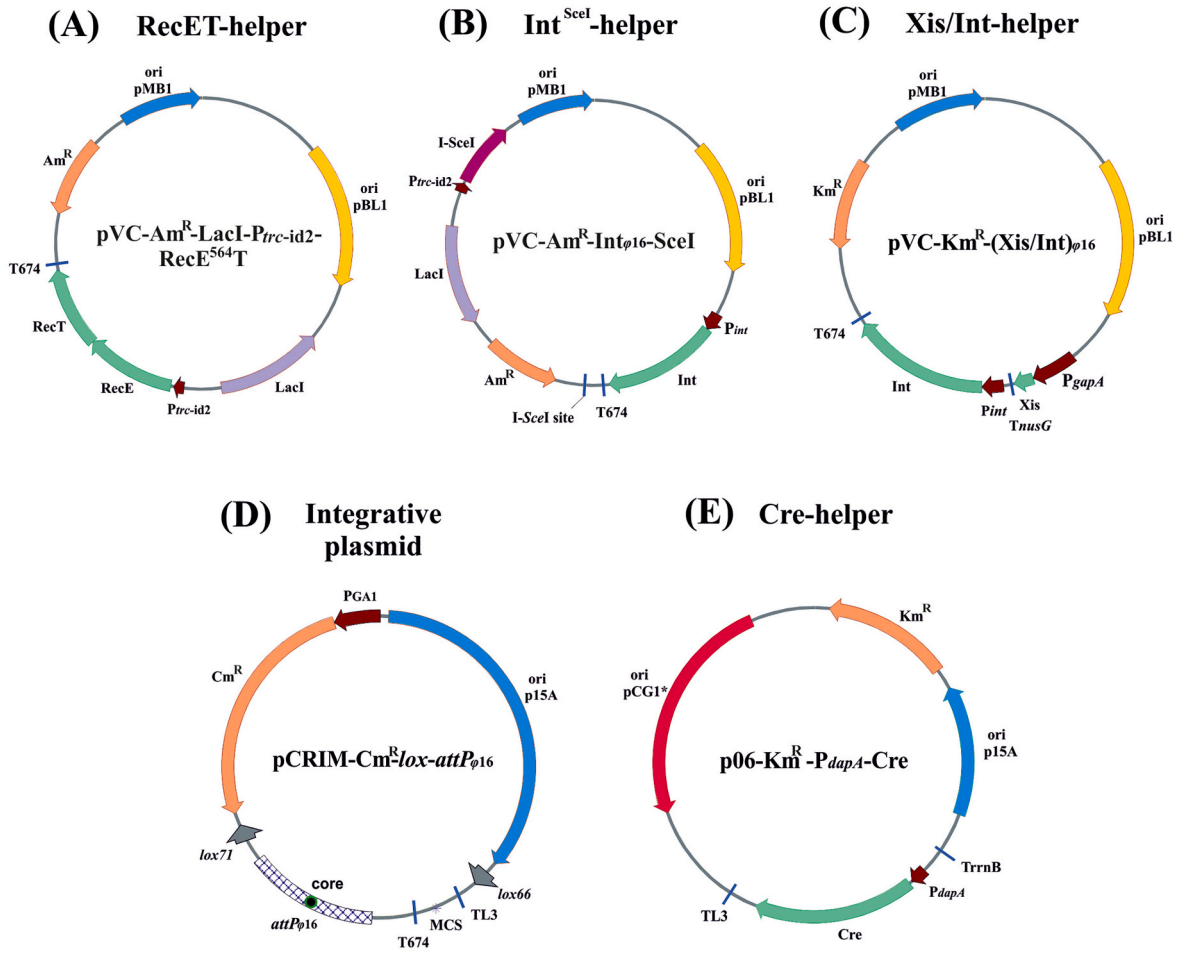

Fig. 2. Schematic map of plasmids for the Dual-In/Out strategy: recombineering helper pVC-Am^R-LacI-P$_{trc\text{-}id2}$-RecE^{564}T(OK651221) (A); integration helper pVC-Am^R-Int$_{\phi16}$-SceI(OK651222) (B);excision helper pVC-Km^R-(Xis/Int)$_{\phi16}$ (OK651223) (C); integrative plasmid pCRIM-Cm^R-*lox-attP*$_{\phi16}$(OK651220) (D); and excision helper p06-Km^R-P$_{dapA}$-Cre (OK651224) (E). Corynephageϕ16 integrase and excisionase genes and *E. coli* Rac prophage RecE^{564}T genes are marked in green, promoters in brown, antibiotic resistance genes in orange, *lox*-sites in grey, *E. coli* origins of replication in blue and *C. glutamicum* pBL1, pCG1 origins of replication inyellow and red, correspondingly. Phage *attP*$_{\phi16}$ is criss-cross hatched. Terminators are indicated with vertical bars, multiple cloning site (MCS) with asterisk. The indicated essential genetic elements of the constructions are described in the text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Lobanova et al., 2017)), Int$_{\phi16}$ and probably several host proteins are necessary.

Thus, replicating plasmids producing solely Int$_{\phi16}$ or both (Int/Xis)$_{\phi16}$ paired proteins were constructed to provide efficient integration or excision processes of targeted DNA molecules according to SSR$_{\phi16}$ mechanisms. Both plasmids were constructed again on the backbone of the pVC7N shuttle vector.

The helper plasmid for integration, pVC-Am^R-Int$_{\phi16}$-*Sce*I (GenBank OK651222, Fig. 2B), carries the ϕ16*int* gene (encoding gp33), which was constitutively expressed under the control of its native promoter to provide SSR function. To facilitate plasmid elimination from the cell, the so-called "conditional suicide" mode (Koob et al., 1994; Pósfai et al., 1997) was realized. More than 50% of colonies obtained after overnight cultivation in IPTG-supplemented medium lost the plasmid, which simultaneously carried the I-*Sce*I restriction site and the I-SceI encoding gene (with codons adjusted for translation in *E. coli* (Colleaux et al., 1986)) under the control of the *lacI*-P$_{trc\text{-}id2}$ operational module (see above).

The helper plasmid for excision, pVC-Km^R-(Xis/Int)$_{\phi16}$ (GenBank OK651223, Fig. 2C), provided constitutive expression of the ϕ16*int* gene, as in the previous plasmid, and ϕ16*xis* (encoding gp47) (Lobanova et al., 2017) under the control of the *C. glutamicum* P$_{gapA}$ promoter.

3.2.3. *CRIM plasmids and a "helper" for vector part excision*

The CRIM vector plasmid pCRIM-Cm^R-*lox-attP*$_{\phi16}$(GenBank OK651220, Fig. 2D) was designed on the basis of a previously engineered plasmid from our laboratory, pSTV-Cm^R-(Int-*attP*)$_{\phi16}$, for ϕ16-based SSR-mediated self-integration into the *attB*$_{\phi16}$ site of the *C. glutamicum* chromosome (Table 1, Fig. S1). Similar to its progenitor, the new CRIM vector carried an MCN p15A-based replicon that could function in *E. coli* but not in *C. glutamicum*, contained the *attP*$_{\phi16}$ site for Int$_{\phi16}$-mediated integration (Moreau et al., 1999a) and the gene *cat* from the Tn*9* transposon as a marker under control of the strong phage promoter P$_{GA1}$ (Pátek et al., 1996) that provided a selectable Cm^R phenotype in *C. glutamicum* cells containing the CRIM vector integrated into an *attB*$_{\phi16}$ site on the chromosome. To prevent transcriptional readthrough from the promoters that could be inserted in the multiple cloning site (MCS) of the vector, the MCS was surrounded by the transcription terminators T$_{L3}$ and T$_{674}$. T$_{L3}$is a well-knownρ-independent (intrinsic) transcription terminator of λ phage (Luk and Szybalski, 1982), and T$_{674}$is a bidirectional ρ-independent terminator previously identified in ϕ674 corynephage (Yomantas et al., 2018). After the recombinant CRIM plasmid integration occurred, the vector part of the plasmid bracketed by the mutant *lox66* and *lox71* sites could be eliminated by Cre-dependent SSR. For this purpose, a plasmid constitutively expressing the *cre* gene from the laboratory collection, p06-P$_{dapA}$-*cre* (Gorshkova

et al., 2018),was modified by substitution of the Cm^R marker for Km^R, which resulted in the construction of a new p06-Km^R-P_{dapA}-Cre plasmid (GenBank OK651224, Fig. 2E), for use in the current study.

3.3. *(Int/Xis)$_{\phi16}$-dependent integration/excision and Cre-dependent excision tests*

To test the main group of constructed plasmids, first, the pCRIM-Cm^R-*lox-attP*$_{\phi16}$ plasmid was integrated into the native *attB*$_{\phi16}$ site (**B** in the mutant strain name) of the ATCC 13869 strain with the help of pVC-Am^R-Int$_{\phi16}$-SceI; this was followed by selection for the desired clones on medium supplemented with Cm and curing the helper plasmid in the plasmid "suicide" mode after IPTG addition, with the final selection of Cm^R and Am^S clones resulting in the strain **69 B::Cm** (Table 1). The insertion did not affect cell growth (data not shown). The efficiency of this Int$_{\phi16}$-dependent "*trans*" integration into the native *attB*$_{\phi16}$ site was approximately 2.8×10^4 transformants per 1 μg DNA, which correlated rather well with the data obtained in our laboratory for "*cis*" integration of the plasmid pSTV-Cm^R-(Int-*attP*)$_{\phi16}$ and with the results earlier published by Trautwetter' group (Moreau et al., 1999a). The proper localization of the CRIM plasmid insertion in the current study was confirmed by PCR with the loci-specific primers presented in Table S1.

Site-specific excision of the integrated CRIM plasmid from the native *attB*$_{\phi16}$ site of the **69 B::Cm** strain which resulted in reconstruction of native *attB*$_{\phi16}$ site in the chromosome was successfully executed due to application of the constructed helper pVC-Km^R-(Xis/Int)$_{\phi16}$ plasmid, resulting in the selection of Km^R clones followed by screening of Cm^S phenotypeon medium supplemented with and without Cm. The efficiency of this excision was greater than 95%.

Chromosomal DNA was purified from the obtained **69 B::Cm** strain and used for electrotransformation into Sm^R **69 35:: [mini-Mu(LER)]** cells (Table 1) according to the developed procedure. A total of 648 ± 73 Cm^R Sm^R clones/20 μg of donor DNA/10^8 surviving cells were obtained after electroporation across three independent experiments. The correct transfer of marked points was verified by PCR analysis with appropriate oligonucleotides (Table S1).

Finally, the Cre-mediated excision of the marked vector part from the integrated CRIM plasmid in the presence of thep06-Km^R-P_{dapA}-Cre plasmid was unambiguously demonstrated for the **69 B::Cm** strain with an efficiency of approximately 95–99%.

Additionally, the same set of experiments was successfully repeated for the well-characterized laboratory strains ATCC 13032 and MB001. These strains were initially used for Int$_{\phi16}$-dependent integration of the pCRIM-Cm^R-*lox-attP*$_{\phi16}$ plasmid into the native *attB*$_{\phi16}$ sites of their chromosomes yielding the strains **32 B::Cm** and **MB001 B::Cm**, respectively (Table 1). The chromosomal DNAs were isolated from obtained integrants for further electrotransformation. The mutant Sm^R strains **32,177:: [mini-Mu(LER)]** and **MB001 837:: [mini-Mu(LER)]** (Table 1), were used as recipients for cognate chromosomal DNA transfer, which resulted in the appearance of 376 ± 62 and 548 ± 83 Cm^R Sm^R clones/20 μg of donor DNA/10^8 surviving cells, respectively. The correct transfer of the marked point was verified by PCR analysis with appropriate oligonucleotides (Table S1).

3.4. *Strains with random locations of attB$_{\phi16}$ site precursors followed by attempts to combine the inserted cassettes in one strain*

To construct a *C. glutamicum* strain collection with different locations of an artificial *attB*$_{\phi16}$ site on the chromosomes, it was initially necessary to eliminate the native *attB*$_{\phi16}$ located in ORF on chromosomes of ATCC 13869 and ATCC 13032 (positions 2,483,498-2,483,526 and 2,565,640-2,565,668, respectively). A DNA fragment 30 bp in length, including *attB*$_{\phi16}$, was precisely deleted in frame from the predicted ORF in both strains by the standard HR-based method with final SacB-contraselection of the targeted recombinants (as repeatedly described, e.g., by Jäger et al., 1992; Schäfer et al., 1994; Tan et al., 2012) with the help of pBS5T-based (Fukui et al., 2006) plasmid pBS5TΔB (Table 1; Supplementary materials). Desired modifications were verified by PCR and amplicon sequencing in the obtained strains and called **69 ΔB** and **32 ΔB**, respectively (Table 1).

For insertions of an artificial *attB*$_{\phi16}$ site into the genome of **69 ΔB**, the targeted recipient strain was initially transformed by the pVC-Am^R-LacI-$P_{trc\text{-}id2}$-$RecE^{564}T$ plasmid for subsequent recombineering with a specifically designed linear dsDNA fragment containing an antibiotic resistance marker (Sm^R or Cm^R) surrounded by the terminators T_{L3}, T_{674} and bracketed by (*attL/R*)$_{\phi16}$ sites for further marker excision by (Int/Xis) $_{\phi16}$-dependent SSR as a the central part that has been extended by approximately 0.8-kb arms homologous to the desired integration point (Fig. S2). The specifically constructed (Supplementary materials) non-replicative in *C. glutamicum* plasmids pUCIDT-Ap^R-L-Sm^R-R or pUCIDT-Ap^R-L-Cm^R-R (GenBank OK651226, OK651225) were used as templates for amplification of the central part of the linear dsDNA fragments.

Three random points in the genome (**542**, **1741**, and **1865**) destroying appropriate ORFs without affecting the viability of cells (data not shown) were initially predesigned as targets for the integration of *attB*$_{\phi16}$ precursors by recombineering (Table 3). The three full-length dsDNA fragments (Fig. S2) carrying the Sm^R marker were successfully constructed using six unique oligonucleotides (Table S1) and used for integration.

All three $RecE^{564}T$-mediated integrations were accomplished with a transformation efficiency of approximately 500–750 clones/2 μg dsDNA fragment/1.1×10^8 surviving cells after electroporation. The proper locations of the integrated cassettes were confirmed by PCR using specially designed primers (Table S1), and chromosomal DNAs of the corresponding strains were isolated to test the possible transfer of the modification by electrotransformation. Surprisingly, none of the three integrated Sm^R markers could be transferred to the **69 B::Cm** (Table 1) strain by the standard electroporation procedure (Table 3).

3.5. *Creation of attB$_{\phi16}$ sites in the points preselected by mini-Mu unit integration*

The aforementioned construction of the *attB*$_{\phi16}$ site precursor was repeated for another three points on the *C. glutamicum* chromosome: positions **2370** (Gorshkova et al., 2018), **400** and **668,** which were earlier determined as the sites available for the mini-Mu unit integration. Previously, for these points, it was shown, that the marked chromosomal DNA fragments from the corresponding strains, **69 *x*:: [mini-Mu(LER)]** $|x \in \{$**400, 668**$\}$, were successfully transferred to strain **69:: Cm** by electrotransformation (Item **1** of the **Results**; Table 2). All DNA constructs and $RecE^{564}T$-mediated recombineering procedures were successfully executed, and three new Cm^R strains with targeted positions for the *attB*$_{\phi16}$ precursor were obtained on the basis of **69 ΔB** and labeled **69 ΔB400::Cm, 69 ΔB668::Cm** and **69 ΔB 2370::Cm** (Table 1). Specific experiments confirmed that these marked precursors could be successfully transferred by electrotransformation to other strains with

Table 3
The efficiency of marked DNA fragment transfer by the chromosomal electrotransformation method.

Integration point name	Inserted cassette	Position in genome	Transfer efficiency, clones/20 μg DNA/10^8 surviving cells
*C. glutamicum*ATCC13869 (GenBank AP017557.2)			
542	*attL*$_{\phi16}$-T_{674}-Sm^R-	542,642	0
1741	T_{L3}-*attR*$_{\phi16}$ (1.8	1,741,544	0
1865	kb)	1,865,443	0
400	*attL*$_{\phi16}$-T_{674}-Cm^R-	400,172	628 ± 77
668	T_{L3}-*attR*$_{\phi16}$ (1.4	668,996	716 ± 81
2370	kb)	2,370,010	527 ± 56

The transfer efficiency is averaged from the results of three experiments.

efficiencies close to those previously detected (see Item **1** of the **Results**) for different integrated **mini-Mu(LER)-**units (Table 3).

Next, the **69 ΔB400::Cm, 69 ΔB668::Cm** and **69 ΔB 2370::Cm** strains were cured of the Cm^R marker by $(Int/Xis)_{\phi16}$-dependent SSR using the pVC-Km^R-$(Xis/Int)_{\phi16}$ plasmid, resulting in three marker-less strains with different locations of unique $attB_{\phi16}$ sites in their chromosomes, **69 ΔB400::B, 69 ΔB668::B** and **69 ΔB 2370::B** (Table 1). These strains were suitable for $Int_{\phi16}$-mediated integration of desired CRIM plasmids.

Two new CRIM plasmids were constructed with pCRIM-Cm^R-*lox*-$attP_{\phi16}$ as a vector by cloning into MCS region of fluorescent protein genes: *yEGFP* from the pKT128 plasmid (Sheff and Thorn, 2004) under the control of the constitutive P_{dapA} (*C. glutamicum*) promoter at the *Mlu*I/*Xho*I sites, and *TurboRFP* from the pTurboRFP-PRL-vector (Evrogen) downstream of the P_{cskA} (*E. coli*) promoter at the *Eco*RV site resulting in the pCRIM-Cm^R-*lox*-$attP_{\phi16}$-G and the pCRIM-Cm^R-*lox*-$attP_{\phi16}$-R plasmids, respectively (Fig. S3A, B).

First, the *yEGFP*-carrier recombinant CRIM plasmid was integrated into the chromosomes of strains **69 ΔB 400::B** and **69 ΔB 2370::B** in an $Int_{\phi16}$-mediated manner. This procedure resulted in the construction of the Cm^R strains **69 ΔB 400::G-Cm** and **69 ΔB 2370::G-Cm** after helper plasmid elimination. Second, the *turboRFP* carrier CRIM plasmid was integrated into **69 ΔB 668::B**, and finally, the **69 ΔB 668::R-Cm** strain was obtained (Table 1).

To combine insertions, the strain **69 ΔB 400::G-Cm** was chosen as an initial recipient. First, the marker-less strain **69 ΔB 400::G** was obtained by Cre-mediated excision (with efficiencies of approximately 97%) of the *lox66/71*-bracketed marked vector part of the integrated recombinant CRIM plasmid with the help of p06-Km^R-P_{dapA}-Cre. At the next stage, Cm-marked genomic DNAs extracted from the strains **69 ΔB 668::R-Cm** and **69 ΔB 2370::G-Cm** were sequentially transferred to the recipient strain **69 ΔB 400::G** by chromosome electrotransformation, selection of Cm^R-transformants, and marker curing before the next step of the experiment, resulting in **69 ΔB 400::G 668::R 2370::G** as the final strain. The proper locations of the integrated cassettes were confirmed by PCR (Table S1). Therefore, a marker-less and plasmid-free derivative of the strain *C. glutamicum* ATCC 13869 was constructed that contained two copies of the *yEGFP* gene and one copy of the *turboRFP* gene integrated into the selected chromosomal loci.

In a similar way, the adjusted Dual-In/Out strategy was applied for *C. glutamicum* ATCC 13032 chromosome editing. As a result, the strain **32 B::G 2393::R** carrying the *yEGFP* gene in the native $attB_{\phi16}$ site and *turboRFP* gene in 2393 positions was constructed.

To confirm the genotype of constructed strains a fluorescence intensity assay was carried out, and the obtained results are presented in Fig. 3. As seen from the figure, the detected level of fluorescence for the strains carrying one copy of the integrated *yEGFP* gene and/or the *turboRFP* gene in their chromosomes were very similar and, practically, did not depend on the point of gene integration or on the presence/absence of the integrated gene encoding another fluorescent protein. In contrast, the strain carrying two integrated copies of the *yEGFP* genes manifested a twofold increased level of fluorescence typical of yEGFP. Therefore, it was experimentally confirmed that each of the obtained strains with a unique and novel $attB_{\phi16}$ site could be a recipient for $Int_{\phi16}$-mediated integration of any CRIM recombinant plasmids constructed in this study with their subsequent possible transfer to another *C. glutamicum* strain or Cre-dependent elimination of the CRIM vector part, if necessary, according to described protocols.

3.6. Broadening the number of sites that could be combined with $RecE^{564}T$-dependent chromosomal electrotransformation

Analysis of current literature data, especially data concerning the temporarily silenced state of large regions of bacterial genomes (for more details, see Discussion), motivated us to test the possible influence of $RecE^{564}T$ expression on the efficiency of HR-driven integration of the electroporated DNA into the *C. glutamicum* chromosome.

It could be supposed that the efficiency of host-encoded HR-mediated integration of the penetrated endogenous DNA into the bacterial chromosome could be significantly enhanced by RecET activity, even in the case of homologous chromosomal fragments which might be temporarily in the "silenced state" due to the formation of complexes with nucleoid-associated proteins (Dillon and Dorman, 2010; Landick et al., 2015; Verma et al., 2019).

According to the literature (Zhang et al., 1998; Fu et al., 2012; Wang et al., 2019), it seemed possible to use not only a full-sized variant of RecE but also a truncated version of RecE, along with RecT, to test this hypothesis. Therefore, it was decided to repeat the electroporation experiments of different marked donor DNAs using the plasmid-carrier strain *C. glutamicum* ATCC 13869/pVC-Am^R-LacI-$P_{trc\text{-}id2}$-$RecE^{564}$Tas the recipient grown under $RecE^{564}T$-expressed conditions.

Initially, the electroporation experiment with the Sm^R marker carrying chromosomal DNA from the **69,198::[mini-Mu(LER)]** strain (which demonstrated the lowest detectable transformation efficiency (Table 2)) was repeated for the new recombination conditions where $RecE^{564}T$ could facilitate the process of the host-encoded HR. The yield of Sm^R transformants significantly exceeded the previous results by a minimum of one order of magnitude (approximately two hundred clones

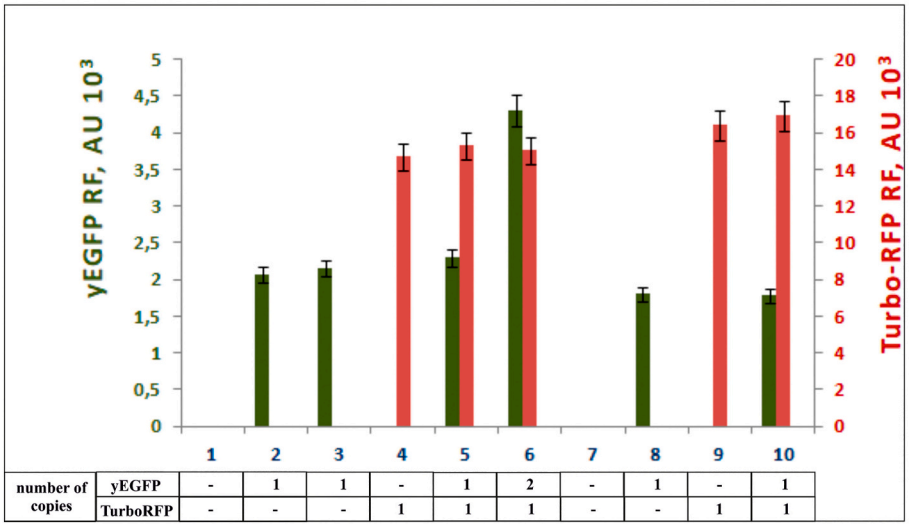

Fig. 3. yEGFP and TurboRFP relative fluorescence intensities in the parental strain ATCC 13869 (1) together with its derivative strains with various combinations of *yEGFP* and *turboRFP* genes, 69 ΔB 400::G (2), 69 ΔB 2370::G (3), 69 ΔB 668::R (4), 69 ΔB 400::G 668::R (5), and 69 ΔB 400::G 668::R 2370::G (6), as well as the parental strain ATCC 13032 (7) together with its derivative strains 32 B::G (8), 32 ΔB 2393::R (9), and 32 B::G 2393::R (10).The copy number of each fluorescent protein gene is indicated in the bottom part under the name of the corresponding strain. Averages of three experiments are shown on graphs, and in all cases, SD does not exceed 15%.

instead of several tens in the comparable experimental conditions (Table 4)). Then, the electroporation experiment was repeated for those Sm^R-marked loci (**542**, **1741** and **1865**) that were not transferred without the pVC-Am^R-LacI-$P_{trc\text{-}id2}$-$RecE^{564}T$ plasmid expression background. Approximately 500–600 Sm^R transformants were positively selected in each experiment based on the new HR conditions facilitated by $RecE^{564}T$ activity (Table 4). The following PCR analysis completely confirmed the expected recombinant DNA structure of the newly obtained clones. It could be supposed that under the conditions of RecET expression, a major portion of the marked *C. glutamicum* chromosomal DNA fragments could be electrotransformed due to significantly enhanced HR. Thus, these donor DNA fragments marked with an antibiotic resistance gene can be transferred into any marker-less *C. glutamicum* strain under the designed conditions. The same approach could be applied for the construction of strains with artificial $attB_{\phi 16}$ sites at new locations as platforms for the targeted insertion of any DNA cassettes of interest.

4. Discussion

The main aim of the present study was adaptation of the Dual-In/Out strategy previously developed for *E. coli*, the advantage of which is the possible introduction of repeated and extended insertions for the broadening of genetic tools for *Corynebacterium glutamicum*. Application of SSR-provided genetic elements of the temperate corynephage ϕ16 in combination with the well-known heterologous elements catalyzed by HR and SSR from the *E. coli* Rac prophage and P1 phage, respectively, helped to develop a new and acceptable version of the desired strategy for chromosomal editing at a predesigned point of the *C. glutamicum* chromosome. A set of specific "helper" and CRIM plasmids was successfully constructed for the *C. glutamicum* system on the basis of autonomously and conditionally replicated vectors, and their desired properties were experimentally confirmed.

Several designed "helpers" were based on the replicon of the shuttle vector pVC7N, which was stable in *C. glutamicum* in selective conditions. To track the "helper" elimination efficiencies of the developed procedure, all "helper" plasmids contained different antibiotic resistance genes, so the initially used MCN "helper" plasmid could be selectively eliminated from *C. glutamicum* cells by introducing a new "helper" with the same replicon but with another marker, followed by overnight cultivation in the presence of the corresponding antibiotic. Moreover, one of the constructed "helpers", pVC-Am^R-$Int_{\phi 16}$-*Sce*I (Fig. 2B), whose elimination is often required at the last stage of a "chromosomal editing" experiment, harbors a gene encoding meganuclease I-SceI and its cognate restriction site. It could be easily self-cured via the "conditional suicide mode", with induced I-SceI expression initiating a double-strand break of the corresponding recognition sequence present in the plasmid

Table 4
The efficiency of marked DNA fragment transfer by the chromosomal electrotransformation method in conditions without and with $RecE^{564}T$expression in recipient cells.

Integration point name	Inserted cassette	Position in genome	Transfer efficiency, clones/20 μg $DNA/10^8$ surviving cells	
			No $RecE^{564}T$ expression	$RecE^{564}T$ expression in recipient cells
*C. glutamicum*ATCC13869 (GenBank AP017557.2)				
198	mini-Mu (LER), Sm^R (3.7 kb)	198,108	16 ± 3	207 ± 25
542	$attL_{\phi 16}$-T_{674}-	542,642	0	506 ± 51
1741	Sm^R-T_{L3}-	1,741,544	0	547 ± 63
1865	$attR_{\phi 16}$ (1.8 kb)	1,865,443	0	619 ± 75

The transfer efficiency is averaged from the results of three experiments.

genome that is correlated well with the known literature data for different bacteria (see, e.g., Volke et al., 2020; Wu et al., 2020).

Therefore, all of the essential steps needed for editing the predesigned point of the *C. glutamicum* chromosome according to the Dual-In/Out strategy were successfully developed and partially optimized. The developed strategy was also successfully applied for integration and further transfer of 10 kb DNA fragment with an efficiency of 150–200 clones/20 μg of donor $DNA/10^8$ surviving cells by the chromosomal DNA electrotransformation method.

However, surprisingly, differences in the ability of chromosomal loci to undergo host-encoded HR with electroporated dsDNA were detected, where only DNA fragments around the point accessible to mini-Mu unit integration could be directly electrotransformed.

Thus, "unsuccessful" points (chosen in Item **4** of the **Results**) could be selected from the extended fragments of the *C. glutamicum* chromosome that manifested a silenced state, probably, due to complex spatial interactions with specific nucleoid-associated proteins, NAPs, called xenogeneic silencers, XS (Navarre, 2016). One of the XS-known assignees, CgpS in *C. glutamicum* (Pfeifer et al., 2016), was able to bind to DNA regions featuring a distinct drop in the GC profile close to the transcription start site (Wiechert et al., 2020) and could play an essential role as a silencer of different genes and cryptic prophage elements whose entrance into the lytic cycle would otherwise cause cell death (Pfeifer et al., 2016; Pfeifer et al., 2019). A member of the *E. coli* XS family, H-NS (the close functional analog of *C. glutamicum* CgpS in the gene silencing process), mediates the formation of higher-order nucleoprotein complexes with AT-rich DNA regions, which results in silencing of the target genes due to the blockade of open complex formation by *E. coli* RNA polymerase with promoters (Spassky et al., 1984), inhibition of RNA synthesis (Liu and Richardson, 1993), or enhancement of termination (Landick et al., 2015). It was also shown that preferable binding of CgpS to AT-rich DNA was important for interference with RNA polymerase and efficient silencing in *C. glutamicum* (Wiechert et al., 2020).

At the same time, it is known that these rather nonspecific but stable NAP-mediated DNA complexes could not prevent highly efficient and sequence-specific DNA-protein interactions, in *E. coli* (Caramel and Schnetz, 1998)and in *C. glutamicum* (Wiechert et al., 2020), leading to the effect of countersilencing.

In keeping with the modern understanding of condition-dependent bacterial nucleoid 3-D structure (Verma et al., 2019), these data served as the basis for the following proposal. It could be supposed that those parts of the *C. glutamicum* chromosome that are temporarily present in the NAP-based silenced complexes could manifest the following: (*i*) a decreased level of host-dependent HR activity with homologous DNA penetrated into the cell by electroporation (that is why several fragments with randomly chosen and marked points could not be integrated by host-encoded HR into the silenced region of the chromosome after electroporation); (*ii*) partial or even full protection from mini-Mu unit transposition (Gorshkova et al., 2018) (such that Mu-driven integration occurs only in the regions of chromosome that are free from the silenced complexes, and freely acceptable for host-encoded HR and the same point marked by Cm^R-precursor of the $attB_{\phi 16}$ site could be successfully electrotransformed); and (*iii*) retention of the ability of RecET-driven HR between the silenced part of the bacterial chromosome and the penetrated linear DNA, which is why the $RecE^{564}T$-driven insertion of any marked precursors of the $attB_{\phi 16}$ site has been successfully obtained even in the silenced regions of the bacterial chromosome, although some of them could not be electrotransformed into the same *C. glutamicum* strain with the standard host-dependent HR conditions. These proposals not only completely explained the obtained experimental results but were also used as the background for the application for *C. glutamicum* Extended-Dual-In/Out-based genome editing strategy.

It is proposed that any marked chromosomal DNA fragment could be transformed into the *C. glutamicum* strain by electroporation if, RecET-encoding genes are overexpressed in the recipient cell to provide an efficient HR between linear penetrated dsDNA and homologous

fragments in the circular cellular chromosome. The experimental data presented above unambiguously confirmed that the expressed pVC-Am^R-LacI-P$_{trc\text{-}id2}$-RecE^{564}T plasmid with a truncated *recE* gene could be applied in the recombineering-based construction of Cm^R-marked *attB*$_{\phi 16}$ precursors due to catalysis of HR between linear dsDNA and replicating circular chromosomes (Items **4** and **5** of the **Results**) and even facilitated and increased the efficiency of HR-dependent electrotransformation of rather extended at least up to 4 kb (Item **1** of the **Results,** Table 2) marked fragments into *C. glutamicum* as a recipient, thus catalyzing the allelic exchange between inserted linear dsDNA and bacterial chromosomes with flank homology (Item **6** of the **Results**).

Hopefully, the developed highly efficient and targeted strategy could be widely used as a convenient genetic tool for genome editing for the construction of different laboratory and industrial *C. glutamicum* strains.

Authors' contribution

Juliya S. Lobanova: Conceptualization, Investigation, Methodology, Writing - Original Draft. **Natalya V. Gorshkova**: Investigation, Validation, Writing - Review & Editing. **Alexander A. Krylov:** Verification, Resources. **Nataliya V. Stoynova:** Supervision, Writing - Review & Editing. **Sergey V. Mashko:** Conceptualization, Writing - Review & Editing.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2022.106555.

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