Additional file 1

Development of a Novel Selection/Counter-selection System for Chromosomal Gene Integrations and Deletions in Lactic Acid Bacteria

Winschau F. Van Zyl, Leon M. T. Dicks[#], Shelly M. Deane

#Correspondence: Leon M. T. Dicks: lmtd@sun.ac.za

Additional file 1: Text S1

Construction of integration vectors

The *L. plantarum* 423 *plaA* bacteriocin gene knockout (KO) plasmid was constructed by PCR, amplifying the complete 2012 bp region of homology that included the *plaA* open reading frame (ORF), using primers plaAKO1 and plaAKO2 (Supplementary Figure S6). The generated amplicon was triple digested with EcoRI, HpaI and XbaI, resulting in the removal of 108 bp from the *plaA* ORF, ligated to a blunt-ended *erm-ffluc* gene cassette and pBluescriptKS digested with EcoRI and XbaI, yielding plasmid pKSplaA::ErmFfluc. The 3204 bp *erm-ffluc* gene cassette contained the *erm* gene for erythromycin resistance, the firefly luciferase gene (*ffluc*) from *Photinus pyralis* fused to the strong constitutive *L. plantarum ldh* gene (Pldh) promoter, generated via PCR using primer pair erm1/fluc2 and plasmid pNZErmFfluc423 (Supplementary Table S1) as source DNA. The pKSplaA::ErmFfluc construct was used as template for PCR amplification of a 5013 bp amplicon containing the *plaA* upstream, *plaA* downstream and *erm-ffluc* gene cassette using primer pair plaAKO1/plaAKO2. Next, the full- length blunt-ended 5013 bp PCR fragment

was cloned into the destination plasmid pNZmazFnisRK**,** before digestion of the plasmid with BglII and filling in the 5' sticky ends, yielding plasmid pNZKOplaA::ErmFfluc.

The pNZKOsrtC::FRTerm plasmid, designed for disruption of the *E. mundtii* ST4SA *sortase C* (*srtC*) gene was constructed as follows. Two regions of homology (~1 kb upstream and ~0.4 kb downstream) flanking the *srtC* gene were PCR amplified using primer pairs srtCKO1for/srtCKO1rev and srtCKO2for/srtCKO2rev, respectively (Supplementary Figure S7). The generated fragments were digested with HindIII/HpaI and HpaI/XbaI, ligated to the blunt-ended FRT-*erm* gene and cloned to the HindIII/XbaI double-digested pBluescriptKS plasmid, yielding plasmid pKSsrtC::FRTerm. Finally, the complete FRT-*erm* –*srtC* and flanking region was amplified from pKSsrtC::FRTerm using primer pair srtCKO1for/srtCKO2rev and cloned into the HindIII-linearized and blunt-ended plasmid pNZmazFnisRK, to yield plasmid pNZKOsrtC::FRTerm.

mCherry reporter gene expression in LAB using the nisin-inducible promoter

To demonstrate the utility of the nisin-induction system using a reporter gene, *Lc. lactis* pNZ9000 transformed with plasmid pNZCherry, *L. plantarum* 423 and *E. mundtii* ST4SA each transformed with plasmid pNZCherrynisRK were grown in MRS or M17 broth for 12 h (Supplementary Table S1). Each 12-h old culture was then serially diluted and plated onto MRS or M17 agar plates supplemented with or without nisin. Expression of the *mCherry* reporter gene was confirmed visually by a colony color change from white to pink or purple. Strains harboring the empty pNZ8048 vector were used as controls.

Detection of _in vitro_ bioluminescence

Lactobacillus plantarum 423 and *E. mundtii* ST4SA recombinant strains carrying the *ffluc* bioluminescence gene were analyzed on agar plates for bioluminescence emission using the

Caliper *in vivo* imaging system (IVIS; Caliper Life Sciences, Hopkinton, MA, USA). Cultures were grown for 12 h in MRS broth, plated onto MRS agar plates using sterile swabs and incubated at 30^{o}C for 24 h. Prior to imaging, one ml of beetle D-luciferin potassium salt substrate (Anatech Instruments, Bellville, South Africa) at 150 µg/ml dissolved in phosphate-buffered saline (PBS) was added directly to the colonies.

Additional file 1: Table S1: Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
Lactobacillus plantarum		
423 pNZ8048	Contains the pNZ8048 plasmid; Cm^R	This study
423 pNZCherrynisRK	Contains the pNZCherrynisRK plasmid; Cm^R	This study
423 bac^-	Derivative of *L. plantarum* 423, cured of the plasmid (pPLA4) harboring the *plaA* bacteriocin gene	[44]
Enterococcus mundtii		
ST4SA pNZ8048	Contains the pNZ8048 plasmid; Cm^R	This study
ST4SA pNZCherrynisRK	Contains the pNZCherrynisRK plasmid; Cm^R	This study
Lactococcus lactis		
pNZ9000 pNZCherry	Contains the pNZCherry plasmid; Cm^R	This study
Listeria monocytogenes		
EGDe	Food-borne clinical pathogen; harbors the pPL2 lux plasmid; Cm^R	Caliper Life Sciences, Hopkinton, MA, U.S.A.
Plasmids		
pNZErmFluc423	pNZ8048 vector carrying the *erm* gene fused to the *Photinus pyralis* firefly luciferase gene (*ffluc*) under the control of the constitutive *L. plantarum* 423 lactate dehydrogenase (*ldh*) gene promoter (Pldh); Em^R, Cm^R	This study
pGKVCatFflucST4SA	pGKV223D vector carrying the *cat* gene fused to the *ffluc* gene under the control of the constitutive *E. mundtii* ST4SA lactate dehydrogenase (*ldh*) gene promoter (Pstldh); Em^R, Cm^R	This study
pKSplaA::ErmFfluc	pBluescriptKS plasmid carrying the *erm-ffluc* gene cassette flanked by *L. plantarum* 423 *plaA* gene regions of homology ; Em^R, Amp^R	This study
pKSmunA::CatFfluc	pBluescriptKS plasmid carrying the *cat-ffluc* gene cassette flanked by *E. mundtii* ST4SA *munA* gene regions of homology; Cm^R, Amp^R	This study
pKSaap::FRTerm	pBluescriptKS plasmid carrying the FRT-flanked *erm* gene flanked by *L. plantarum* 423 *aap* gene regions of homology ; Em^R, Amp^R	This study

pKSsrtA::FRTerm	pBluescriptKS plasmid carrying the FRT-flanked *erm* gene flanked by *E. mundtii* ST4SA *srtA* gene regions of homology ; Em^R, Amp^R	This study
pKSsrtC::FRTerm	pBluescriptKS plasmid carrying the FRT-flanked *erm* gene flanked by *E. mundtii* ST4SA *srtC* gene regions of homology; Em^R, Amp^R	This study
pNZCherry	pNZ8048 vector carrying the *mCherry* fluorescence gene under the control of the PnisA promoter; Cm^R	This study
pNZCherrynisRK	pNZnisRK vector carrying *mCherry* fluorescence gene under the control of the PnisA promoter; Cm^R	This study

Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance; Amp^R, ampicillin resistance

References

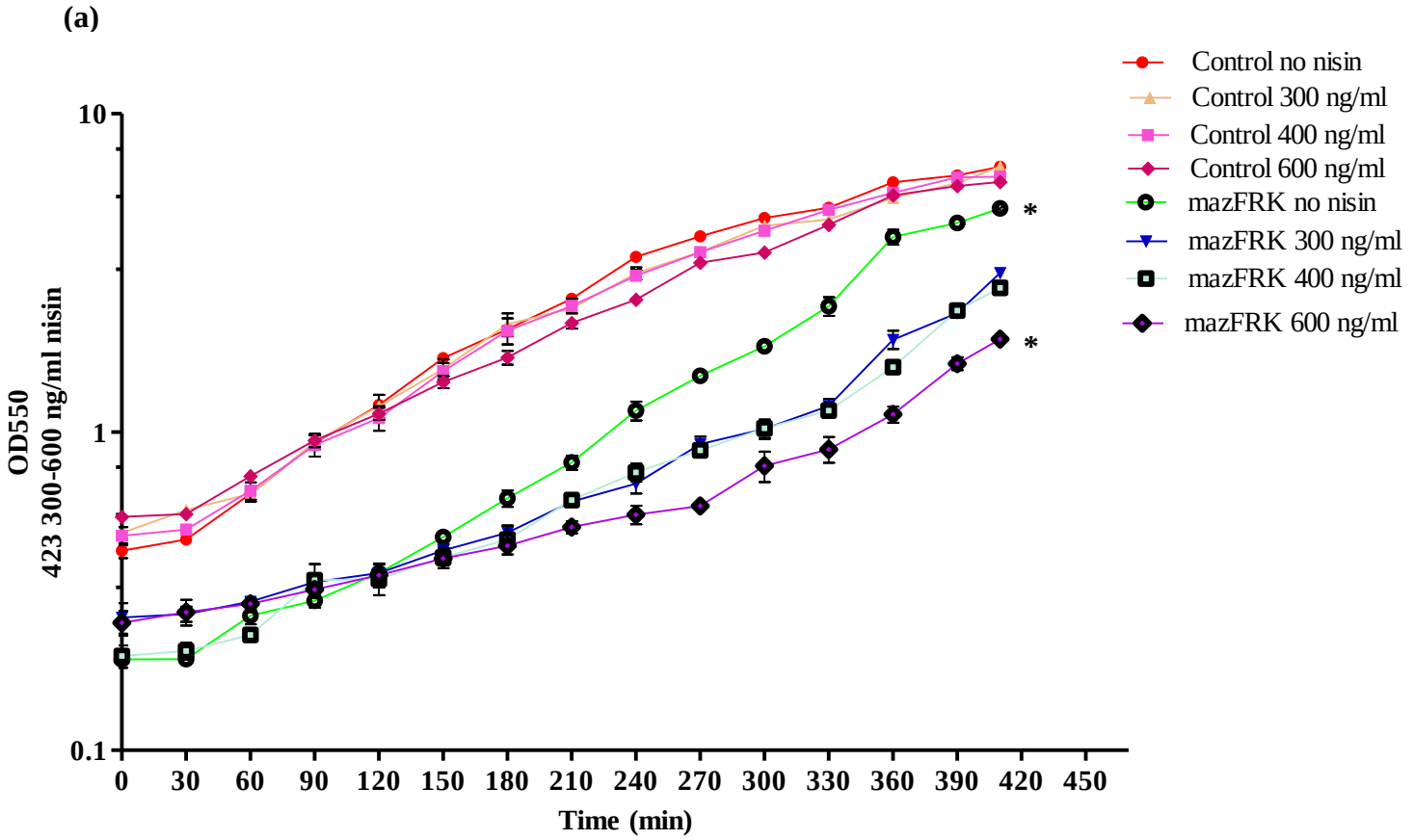
Van Reenen, C.A., Van Zyl, W.H. & Dicks, L.M.T. (2006) Expression of the immunity protein of plantaricin 423, produced by *Lactobacillus plantarum* 423, and analysis of the plasmid encoding the bacteriocin. *Appl. Environ. Microbiol*. 72, 7644–7651.

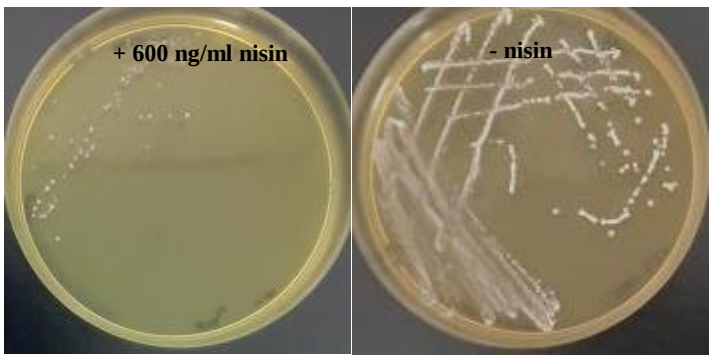
Additional file 1: Table S2: Oligonucleotides utilized in this study

Target	Primer	Sequence (5' to 3')[†]	Restriction sites	Product size (bp)
nisR-nisK	nisRK1	GCGC**AAGCTT**CCCCGGCTTTAGGTATAG	HindIII	2162
	nisRK2	ATCC**CTCGAG**TTACTTTTTTATTTTTAGGATA	XhoI	
mazF	mazF1	GCTTGGAT**CCATGG**TAAGCCGATACGTACC	NcoI	342
	mazF2	AGTC**AAGCTT**CTACCCAATCAGTACGTTAATT	HindIII	
erm-ffluc gene cassette	erm1	ATAC**GAATTC**CACGACCAAAACTATAAAACC	EcoRI	3204
	fluc2	GCGC**AAGCTT**CACAATTTCGACTTGCCA	HindIII	
plaA KO region	plaAKO1	**GAATTC**GTGATATATAATGATGGAATTTATTC	EcoRI	2012
	plaAKO2	**TCTAGA**GCCTTAGCGTACTTATCCCAGGC	XbaI	
munA KO region	munAKO1	GCCG**GAATTC**GTTTATACGATTAGTGGAATTA	EcoRI	1809
	munAKO2	ATAC**TCTAGA**GACAATATCTCCAGTTTT	XbaI	
cat-ffluc gene cassette	cat1	G**GAATTC**GGATCCATTCTAATGAAG	EcoRI	2349
	fluc2	GCGC**AAGCTT**CACAATTTCGACTTGCCA	HindIII	
aap KI region	aapKO1	GCCG**GAATTC**GAGTACTGTGACGACAGCTA	EcoRI	1848
	aapKO2	ATAC**TCTAGA**TAATCGTCCGGGTAACCGTT	XbaI	
FRT-*erm* gene	M13for	GTAAAACGACGGCCAG	blunt	1458
	M13rev	CAGGAAACAGCTATGA	blunt	
srtA upstream region	srtAKO1for	CACC**AAGCTT**GTAGGTTACTTCATCAACGG	HindIII	1011
	srtAKO1rev	GATA**GTTAAC**TAGTAGAAACAATAGGAAAT	HpaI	
srtA down-stream region	srtAKO2for	TATA**GTTAAC**AAAGAAGCCACCCAAGAAAT	HpaI	376
	srtAKO2rev	GATA**TCTAGA**GCCGAAAAATTTACTGATGA	XbaI	
srtC upstream region	srtCKO1for	GAGG**AAGCTT**ATTAGATGGGCAAGACGTAG	HindIII	1011
	srtCKO1rev	GATA**GTTAAC**CCGATAAAAAATACCGAGAA	HpaI	
srtC down-stream region	srtCKO2for	CACC**GTTAAC**TTTTTGTTGCTATTTCTTAT	HpaI	1035
	srtCKO2rev	CACC**TCTAGA**AGATTATAATTTGAATTTTA	XbaI	

FRT target 1	FRTfor1	AGAT**GGATCC**CTCGTTTTCGGAAACGCTTT	BamHI	121
	FRTrev1	GATA**CTGCAG**TTCAGAGCGCTTTTGGTTTT	PstI	
FRT target 2	FRTfor2	AGAT**AAGCTT**CTCGTTTTCGGAAACGCTTT	HindIII	121
	FRTrev2	GATA**GTCGAC**TTCAGAGCGCTTTTGGTTTT	SalI	
erm gene	erm1	ATAC**GAATTC**CACGACCAAAACTATAAAACC	EcoRI	1063
	erm 2	GCTT**GAATTC**TTACTTATTAAATAATTTATAG		
FLP gene	FLPfor	GATA**CCATGG**ATGCCACAATTTGGTATATT	NcoI	1293
	FLPrev	AGTA**GTCGAC**TTATATGCGTCTATTTATGT	SalI	
asRNA repA	asRNAfor	ATAT**CCATGG**TTCATATGAACCTTTGAT	NcoI	350
	asRNArev	CACG**AAGCTT**GATAAGGTAATTATATCAT		
Pldh-FLP amplicon	Pldh1	**GAATTC**AATCTTCTCACCGTCT	EcoRI	1794
	FLPrev	AGTA**GTCGAC**TTATATGCGTCTATTTATGT	SalI	
plaA KO integration region	plaAKOc	AATATCTTCGTTGCTGTGAT	-	5118
	plaAKO2	**TCTAGA**GCCTTAGCGTACTTATCCCAGGC	XbaI	
munA KO intergratio n region	munAKOc	ATTCTTGAGAACATTCCACA	-	4190
	munAKO2	ATAC**TCTAGA**GACAATATCTCCAGTTTT	XbaI	
aap KI integration region	aapKOc	GGCGCCAGCAGCCAACTCAA	-	3696
	aapKOc2	CGTAGCCCTCATGGCTCAGA	-	
aap KI unmarked integration region	aapKOc	GGCGCCAGCAGCCAACTCAA	-	2573
	aapKOc2	CGTAGCCCTCATGGCTCAGA	-	
s*rtA* KO integ ration region	srtAKOc	GATGGTTTTGTTTATTCGAA	-	3853
	srtAKO2rev	GATATCTAGAGCCGAAAAATTTACTGATGA	-	
srtC KO integration region	srtCKOc	GAAGGAACGCTGAAGGTCAA	-	3183
	srtCKOc2	AAACTAGTCCTGTCGTTCCTT	-	

†: Bold and underlined sequences indicate restriction sites; Full target gene names are listed in main text and Table 2; KO: knockout; KOc: knockout confirmation; for: forward; rev: reverse.

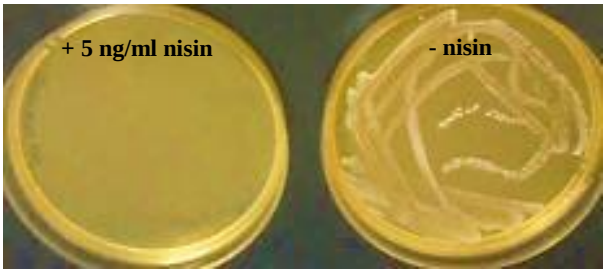




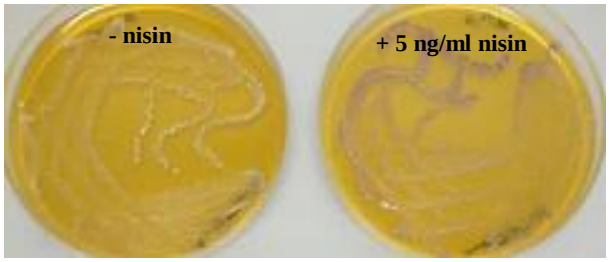
Additional file 1: Fig. S1. Optimization of nisin-controlled *maz*F gene expression in *L. plantarum* 423. **(a)** Growth comparison of *L. plantarum* 423 transformed with the empty pNZ8048 vector (control) and *L. plantarum* 423 transformed with the PnisA controlled *maz*F gene pNZmazFnisRK plasmid in sub-inhibitory concentrations of nisin (0 - 600 ng/ml). Significant differences ($P<0.05$; Kruskal-Wallis nonparametric test) between mazFRK no nisin and mazFRK 600 ng/ml are

indicated with an asterisk. **(b)** MRS agar plates representative of the effect of MazF protein expression in *L. plantarum* 423 harboring the pNZmazFnisRK plasmid in the absence of nisin (-nisin) and in the presence of nisin (+600 ng/ml nisin).

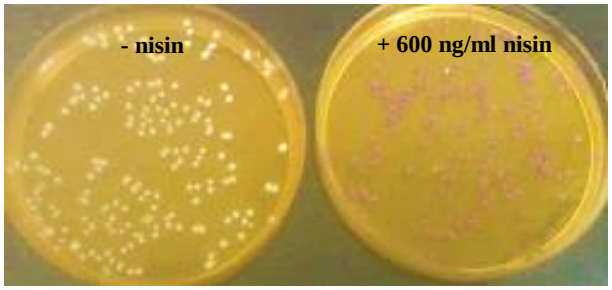
(a)



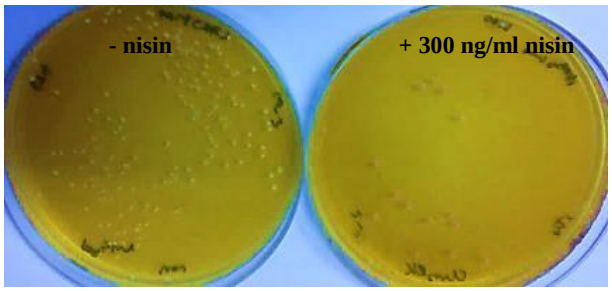
(b)



©



(d0

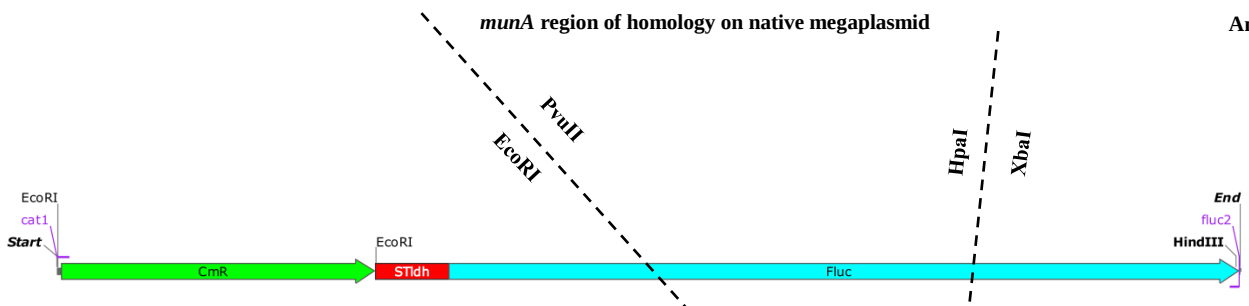


Additional file 1: Fig. S2. Plates showing the effect of PnisA promoter-controlled MazF protein expression in *Lc. lactis* pNZ9000 and PnisA promoter-controlled mCherry fluorescence protein expression in *Lc. lactis* pNZ9000, *L. plantarum* 423 and *E. mundtii* ST4SA. **(a)** M17 agar plates representative of the effect of MazF protein expression in *Lc. lactis* pNZ9000 harboring the pNZmazF plasmid in the absence of nisin (-nisin) and in the presence of nisin (+5 ng/ml nisin). **(b)** M17 agar plates showing the expression of the *mCherry* fluorescence gene in *Lc. lactis* pNZ9000 harboring the pNZCherry plasmid in the absence of nisin (-nisin) and in the presence of nisin (+5 ng/ml nisin). **(c)** MRS agar plates showing the expression of the *mCherry* fluorescence

gene in *L. plantarum* 423 harboring the pNZCherrynisRK plasmid in the absence of nisin (-nisin) and in the presence of nisin (+600 ng/ml nisin). **(d)** MRS agar plates showing the expression of the *mCherry* fluorescence gene in *E. mundtii* ST4SA harboring the pNZCherrynisRK plasmid in the absence of nisin (-nisin) and in the presence of nisin (+300 ng/ml nisin).

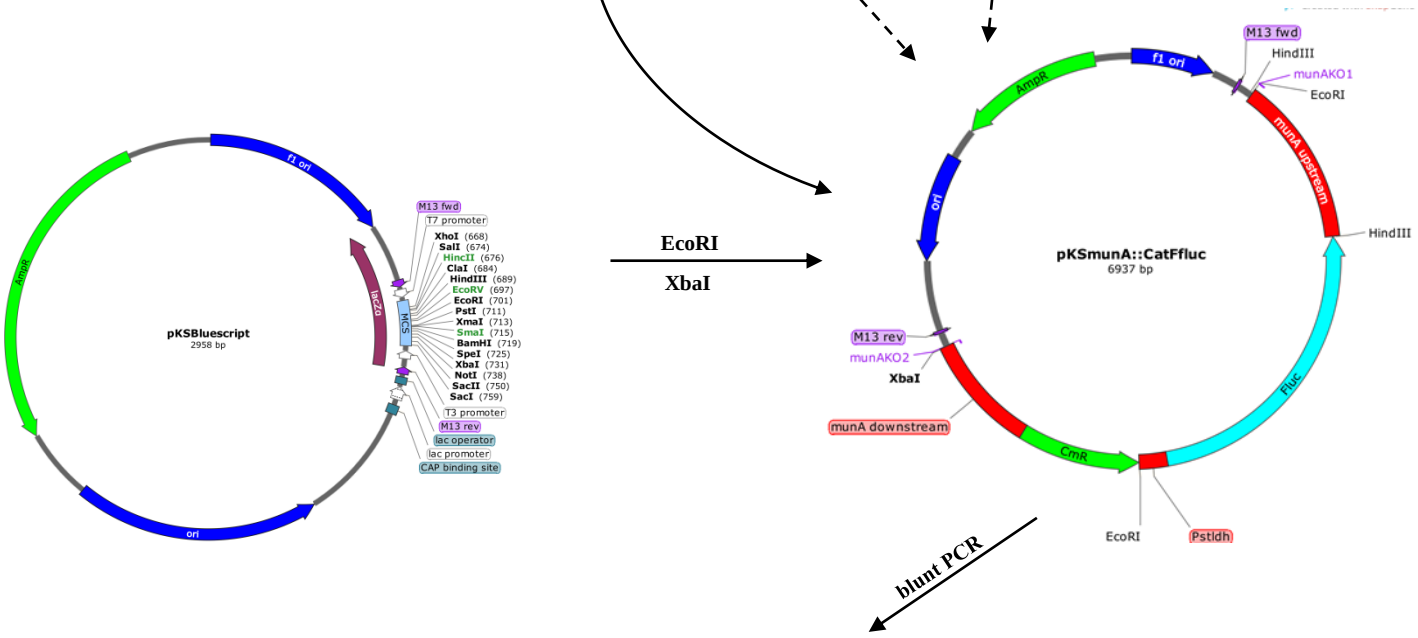
***munA* region of homology on native megaplasmid**

Amplicon size: ~ 1.8 kb

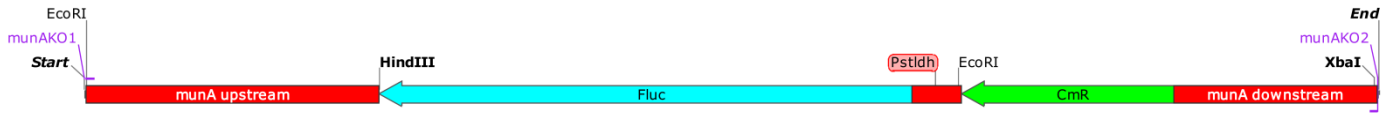


***cat-ffluc* blunt PCR**

Amplicon size: ~ 2.4 kb

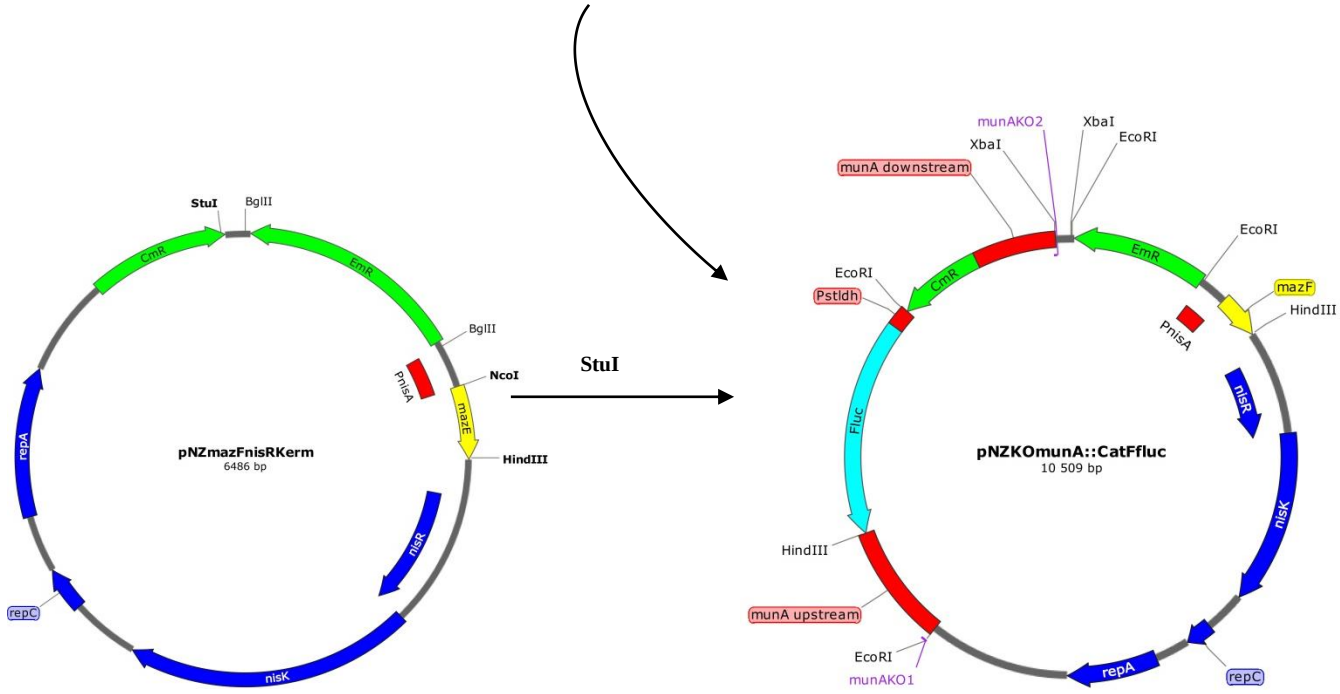


blunt PCR

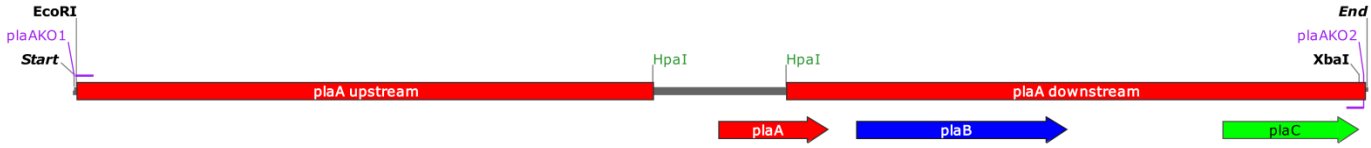


Amplicon size: ~ 4 kb

***munA::catffluc* amplicon**

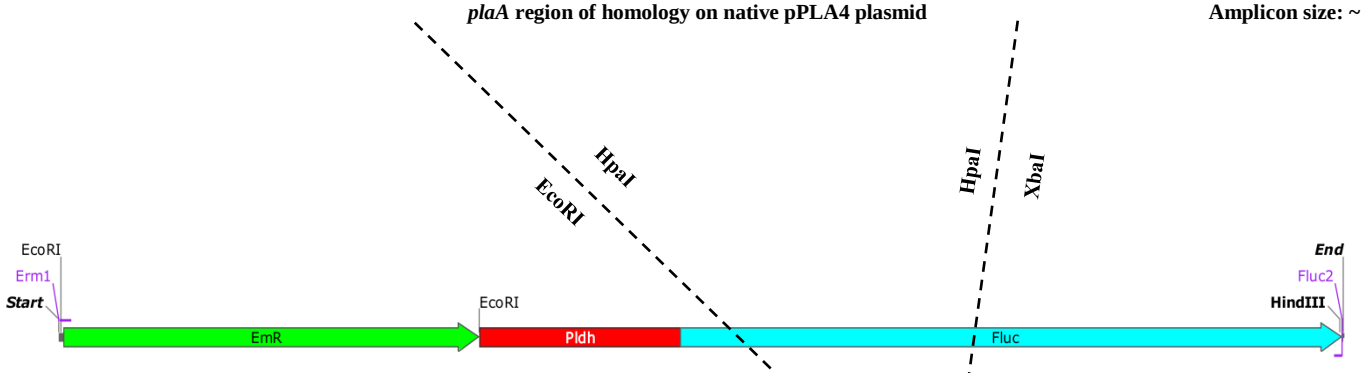


Additional file 1: Fig. S3. Schematic representing the construction of the pNZKOmunA::CatFfluc integrative plasmid containing the *cat* gene and two flanking sequences of the *munA* bacteriocin gene from *E. mundtii* ST4SA. Relevant genes, restriction sites and PCR primers are shown. Construction of the plasmid is best understood by referring to "Materials and Methods" in the main text.



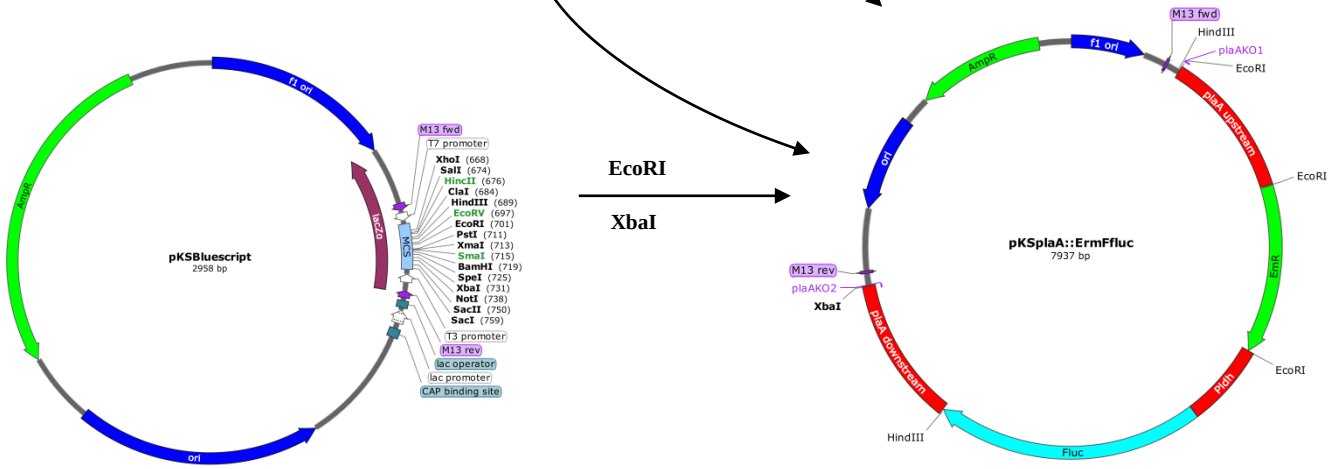
***plaA* region of homology on native pPLA4 plasmid**

Amplicon size: ~ 2 kb



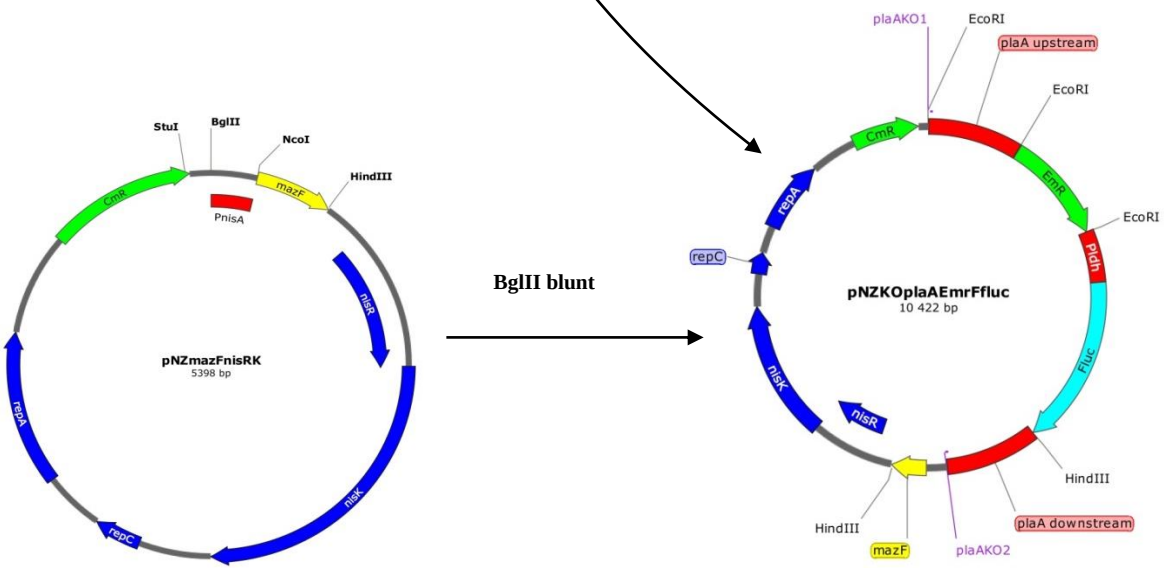
***erm-ffluc* blunt PCR**

Amplicon size: ~ 3.2 kb

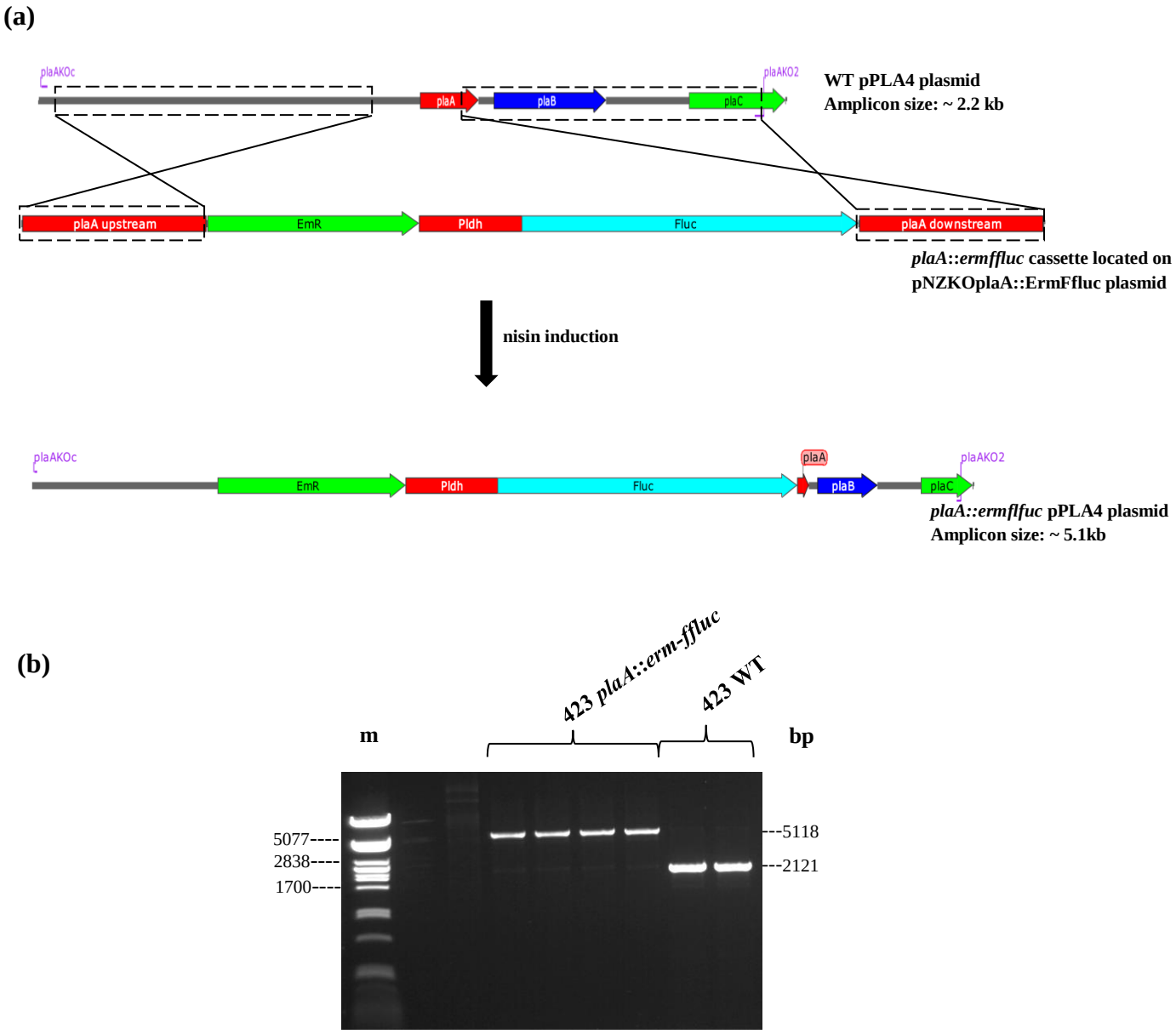


***plaA::ErmFfluc* amplicon**

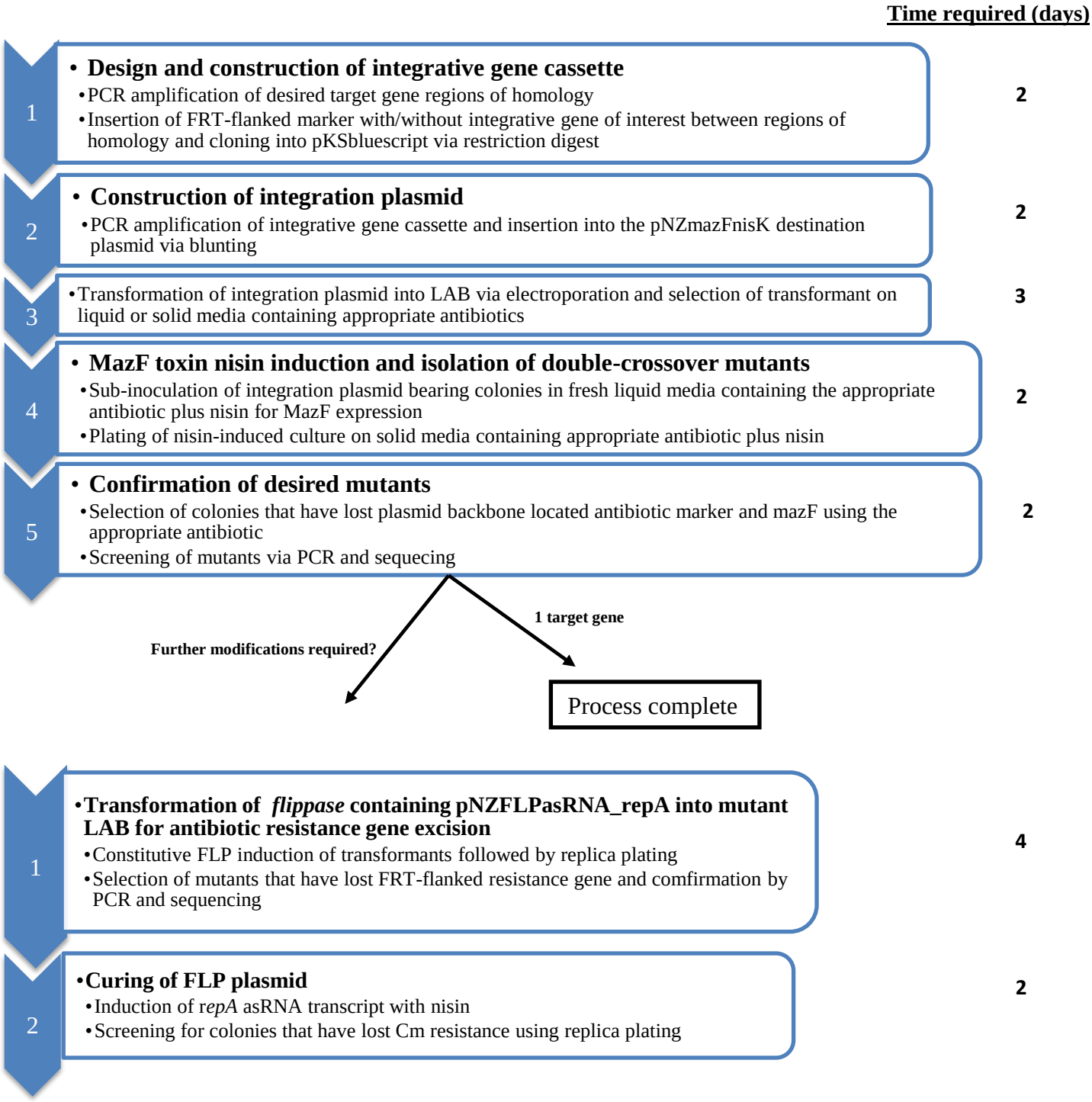
Amplicon size: ~ 5 kb



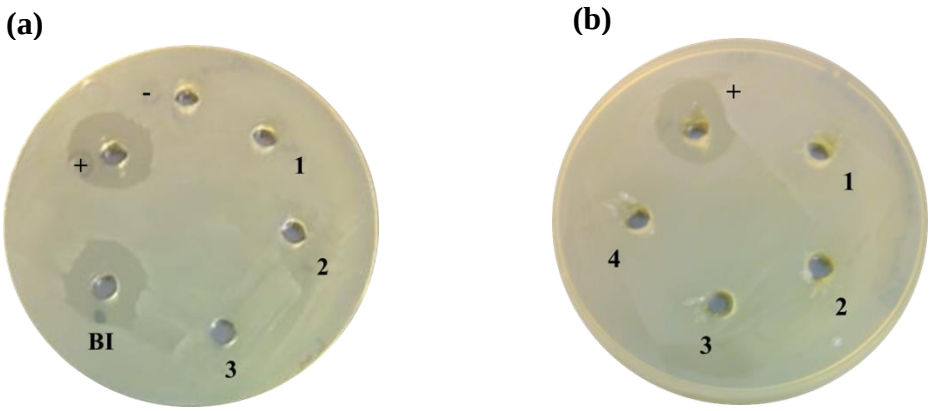
Additional file 1: Fig. S4. Schematic representing the construction of the pNZKOplaA::ErmFfluc integrative plasmid containing the *erm* gene and two flanking sequences of the *plaA* bacteriocin gene from *L. plantarum* 423. Relevant genes, restriction sites and PCR primers are shown. Construction of the plasmid is best understood by referring to the Additional file 1 Text S1.



Additional file 1: Fig. S5. Gene deletion and integration via homologous recombination into the genome of *L. plantarum* 423 at the *plaA* bacteriocin gene locus to create *L. plantarum* 423 *plaA::erm-ffluc*. **(a)** Homologous recombination between the wild-type (WT) pPLA4 plasmid and the *plaA::ermffluc* cassette. Boxed regions show the upstream and downstream regions of homology (~ 0.9 kb) on plasmid pPLA4 and the pNZKOplaAErmFfluc KO vector. Cells harboring the *plaA* KO vector were selected on Cm and Em, followed by nisin induction for MazF toxin expression to select for mutants that have lost the plasmid backbone bearing *cat* and *mazF* genes. Double crossover mutants were selected and screened by PCR using the primer combinations indicated in purple. **(b)** PCR amplification of WT and *plaA* deletion and insertion mutants using the primer pair indicated in panel A. **(m)** Lambda DNA digested with PstI (NEB). Amplicons from four *plaA* mutant and two WT colonies, respectively, are shown.

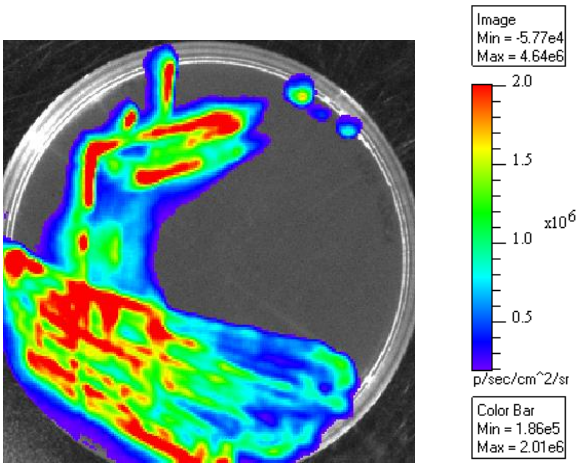


Additional file 1: Fig. S6. A workflow diagram showing the step-by-step design and protocol of the newly developed counterselection method for the deletion and inactivation of LAB genes or to introduce genes of interest followed by resistance marker recycling.

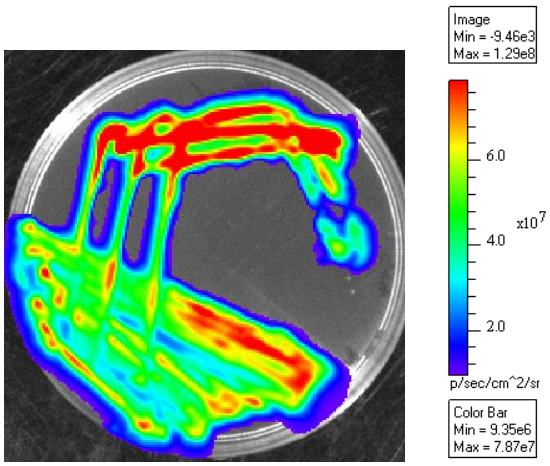


Additional file 1: Fig. S7. Zones of inhibition on plates overlaid with *L. monocytogenes* EGDe. **a.** (BI) Supernatant containing bacteriocin plantaricin 423 isolated from *L. plantarum* 423 transformed with KO plasmid pNZKOplaA::ErmFfluc before induction with nisin, (+) supernatant containing bacteriocin plantaricin 423 isolated from wild-type (WT) *L. plantarum* 423 as positive control, (-) supernatant lacking plantaricin 423 bacteriocin isolated from *L. plantarum* 423 bac- as negative control and supernatants (1, 2 & 3) lacking bacteriocin plantaricin 423 isolated from three *L. plantarum* 423 *plaA::erm-ffluc plaA* gene deletion mutants. **b.** (+) Supernatant containing bacteriocin mundticin ST4SA isolated from WT *E. mundtii* ST4SA as positive control and supernatants (1, 2, 3 & 4) lacking bacteriocin mundticin ST4SA isolated from four *E. mundtii* ST4SA *munA::cat-ffluc munA* gene deletion mutants.

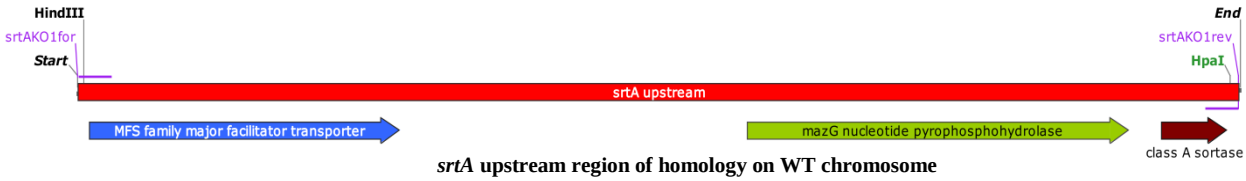
(a)



(b)

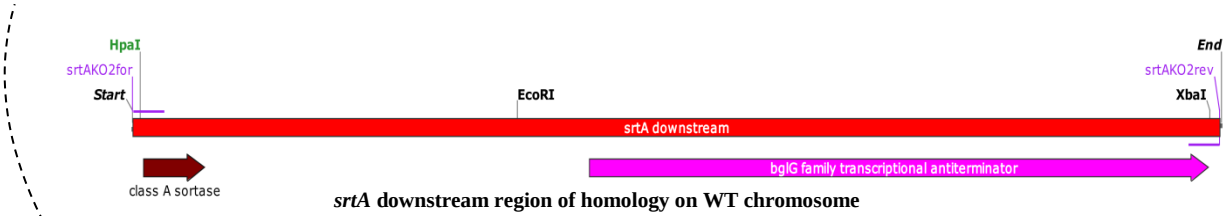


Additional file 1: Fig. S8. MRS agar plates showing bioluminescence emission of *ffluc* luciferase gene integration mutants. **(a)** Bioluminescent *L. plantarum* 423 *plaA::erm-ffluc* double-crossover mutant colonies harboring the *ffluc* gene integrated at the *plaA* locus. (b) Bioluminescent *E. mundtii* ST4SA *munA::cat-ffluc* double-crossover mutant colonies harboring the *ffluc* gene integrated at the *munA* locus. Images were generated using the Living Image® software program and representative scale bars (in photons per second) are indicated for each image (red, most intense and purple being the least intense).



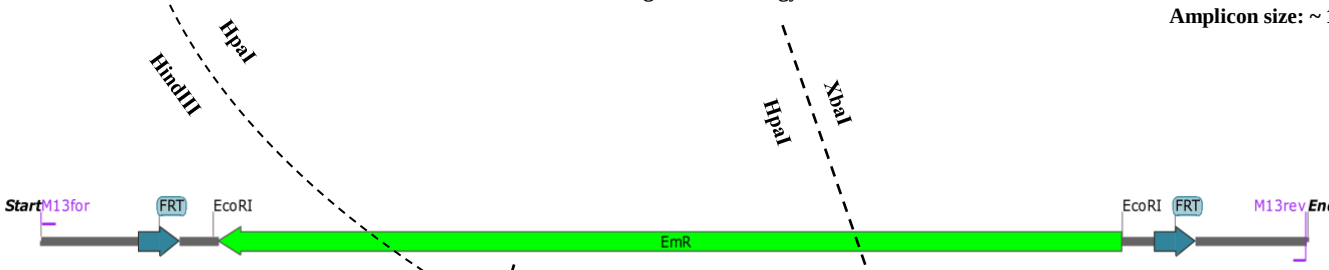
***srtA* upstream region of homology on WT chromosome**

Amplicon size: ~ 1 kb



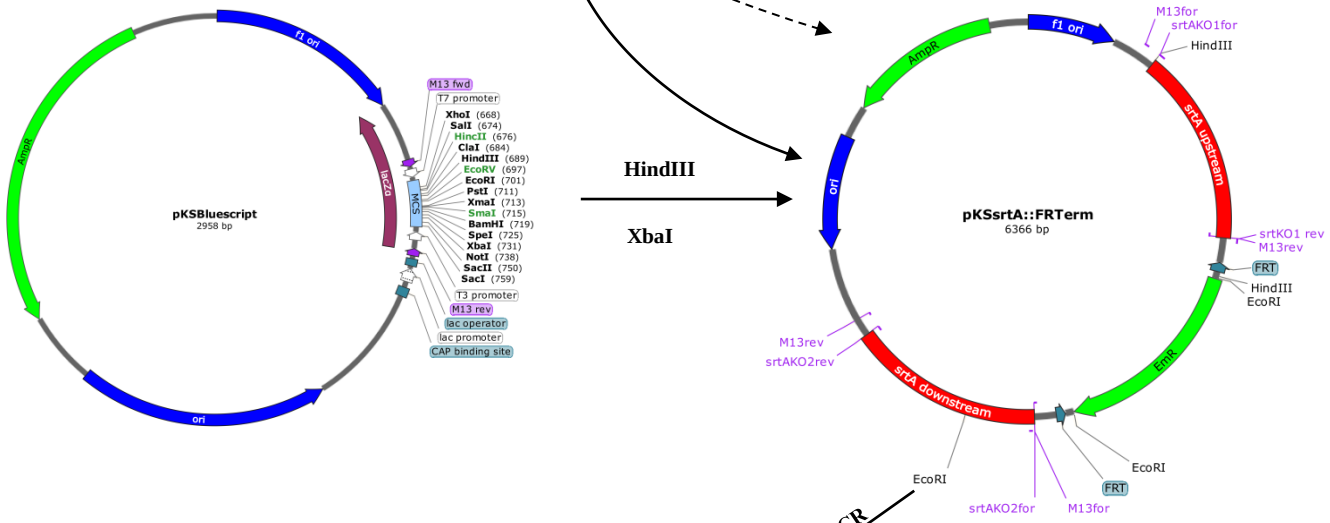
***srtA* downstream region of homology on WT chromosome**

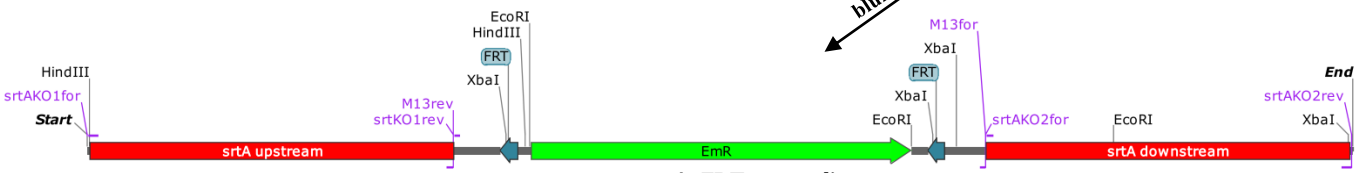
Amplicon size: ~ 1 kb



FRT-flanked *erm* blunt PCR

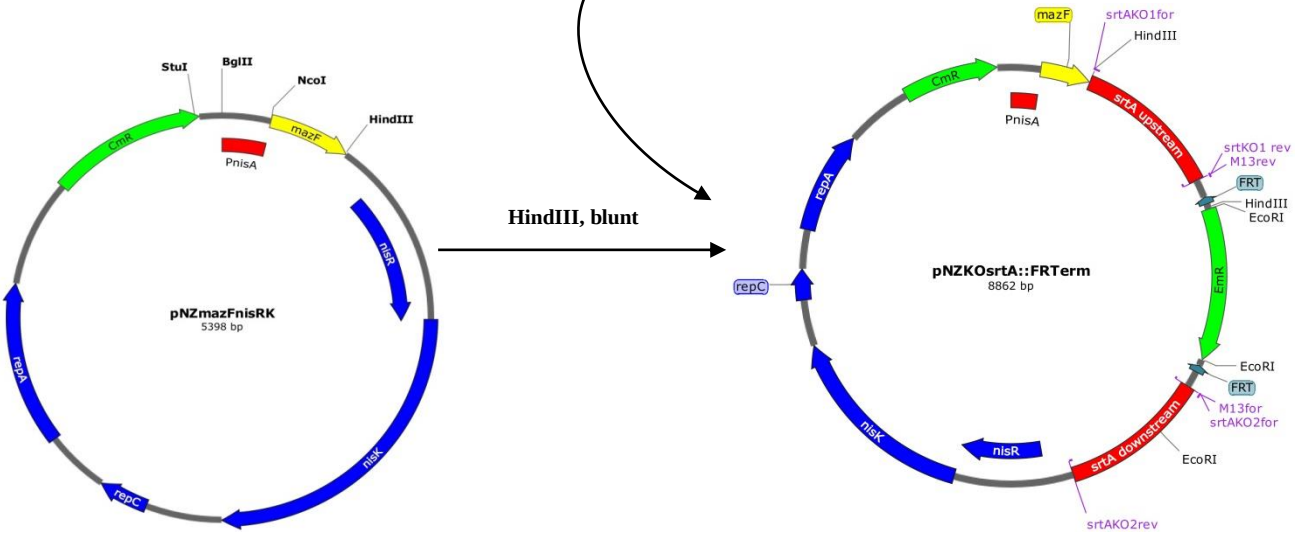
Amplicon size: ~ 1.5 kb



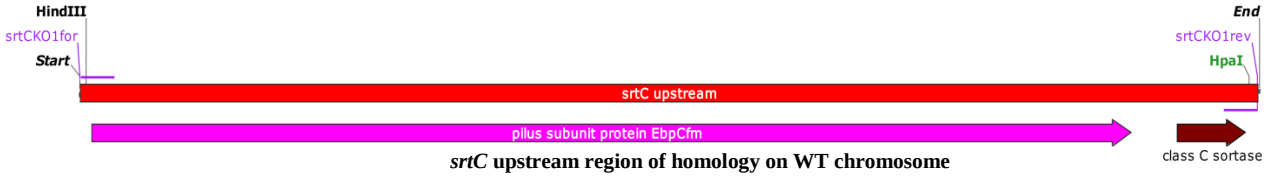


***srtA*::FRT*erm* amplicon**

Amplicon size: ~ 3.5 kb

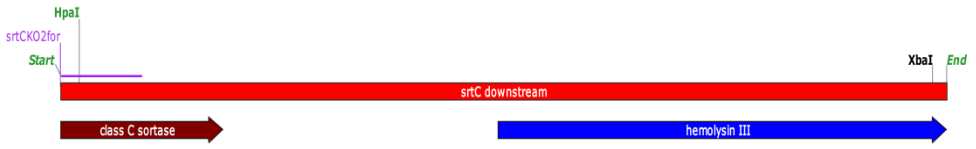


Additional file 1: Fig. S9. Schematic representing the construction of the pNZKOsrtA::FRTerm integrative plasmid containing the *erm* gene and two flanking sequences of the *srtA* cell wall adhesion associated gene from *E. mundtii* ST4SA. Relevant genes, restriction sites and PCR primers are shown. Construction of the plasmid is best understood by referring to "Materials and Methods" in the main text.



srtC **upstream region of homology on WT chromosome**

Amplicon size: ~ 1 kb

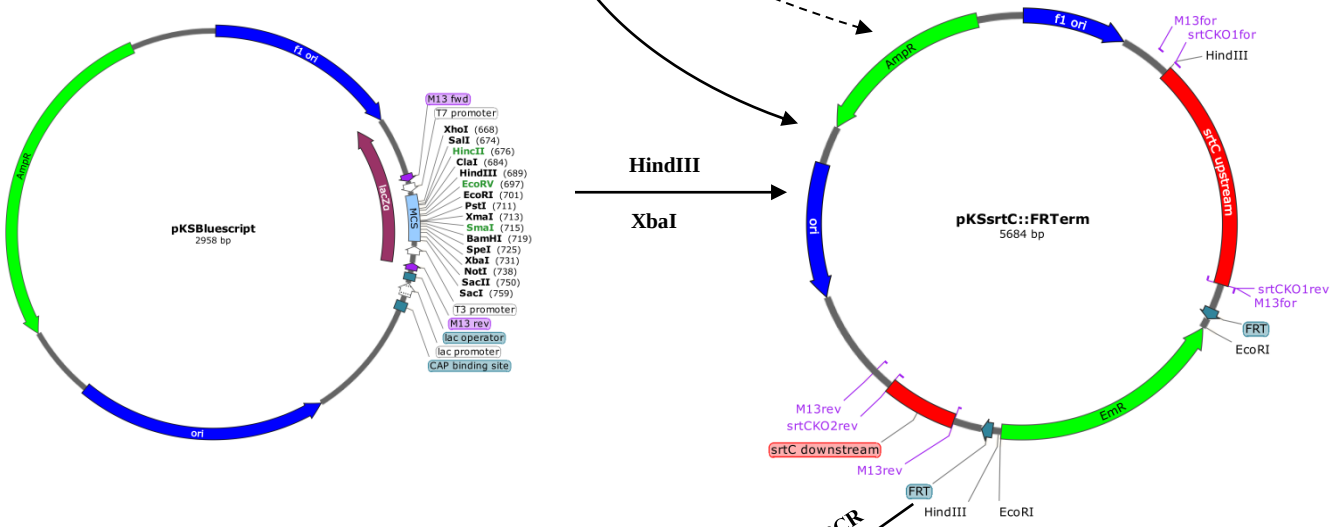


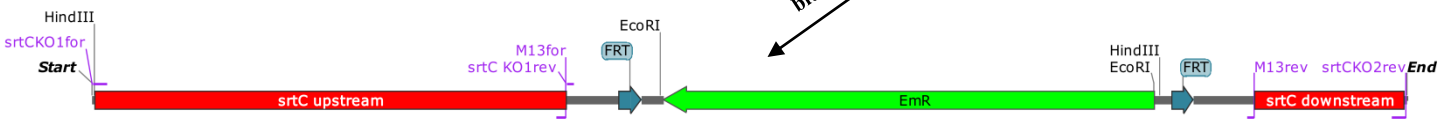
srtC **downstream region of homology on WT chromosome**

Amplicon size: ~ 0.4 kb

FRT-flanked *erm* blunt PCR

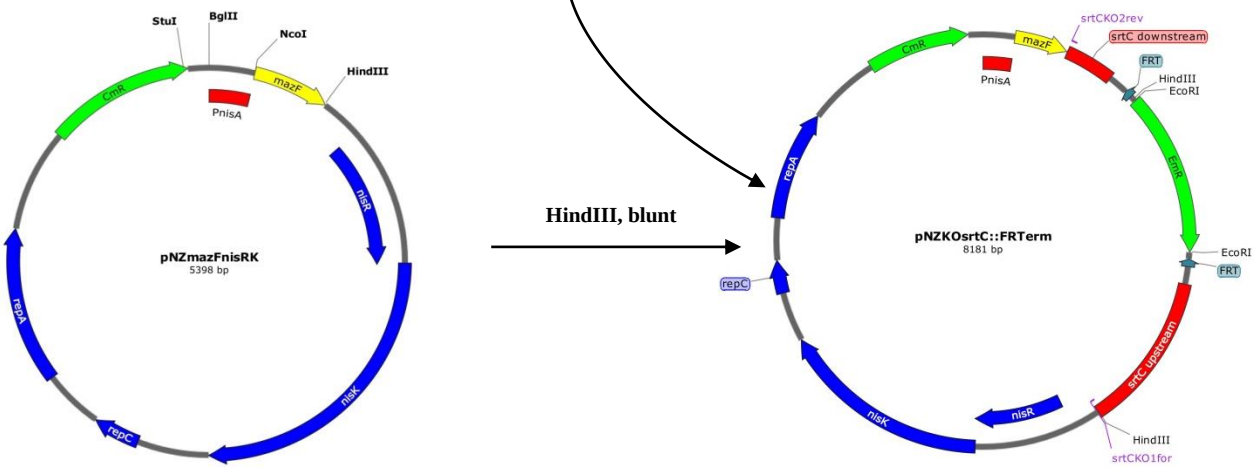
Amplicon size: ~ 1.5 kb





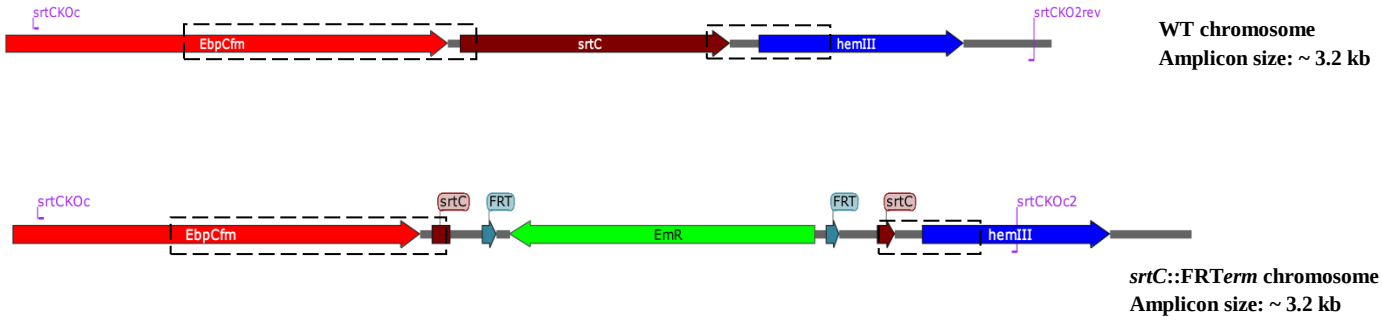
srtC**::FRT*****erm*** **amplicon**

Amplicon size: ~ 2.8 kb

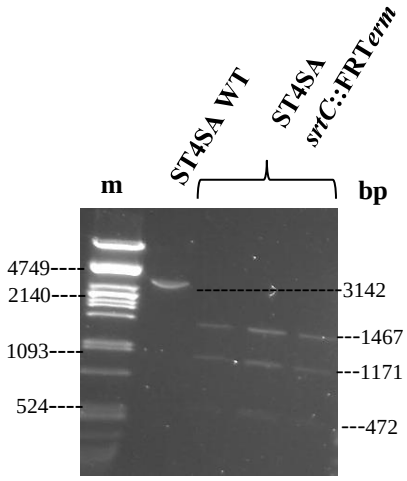


Additional file 1: Fig. S10. Schematic representing the construction of the pNZKOsrtC::FRTerm integrative plasmid containing the *erm* gene and two flanking sequences of the *srtC* cell pilus associated gene from *E. mundtii* ST4SA. Relevant genes, restriction sites and PCR primers are shown. Construction of the plasmid is best understood by referring to the Additional file 1 Text S1.

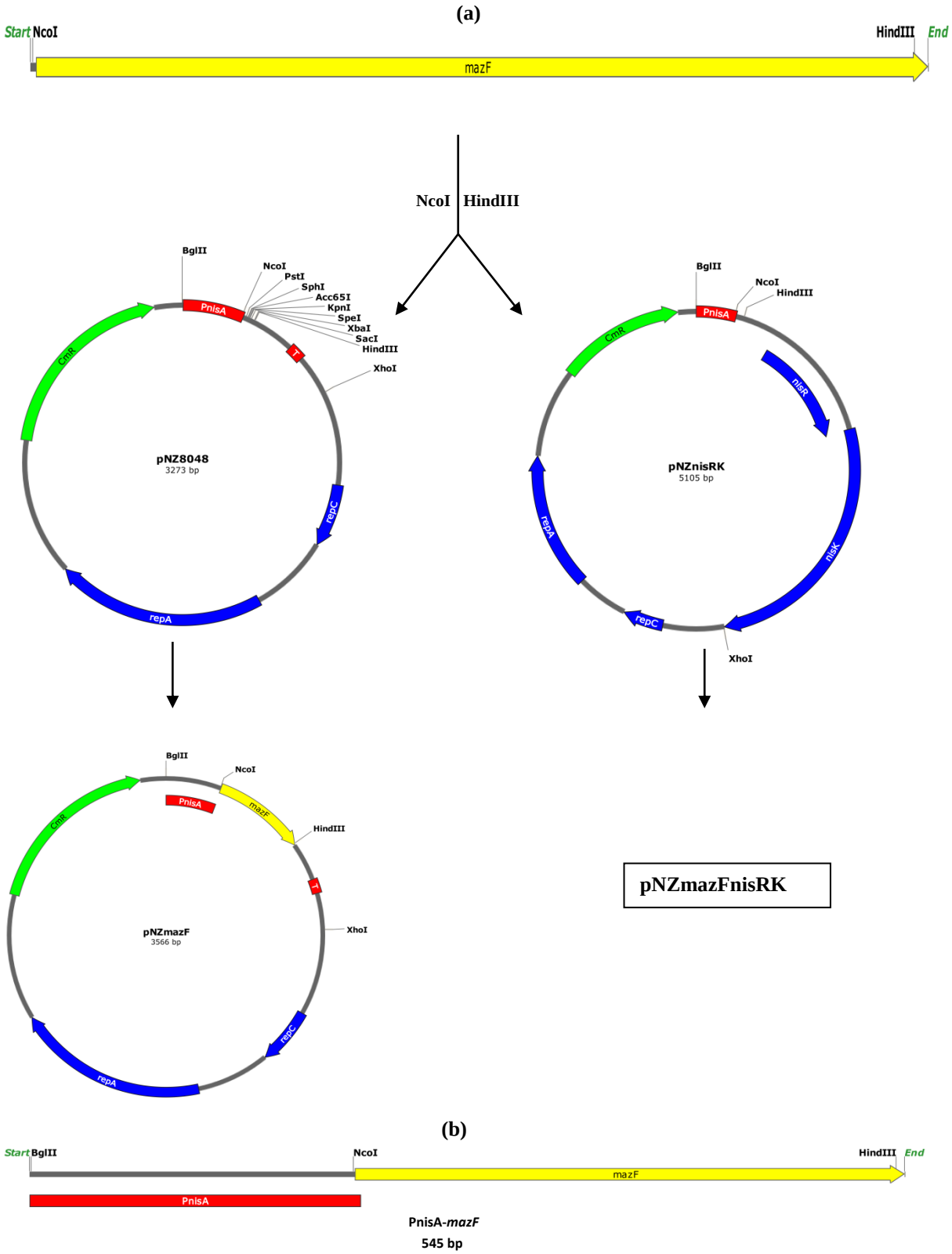
(a)



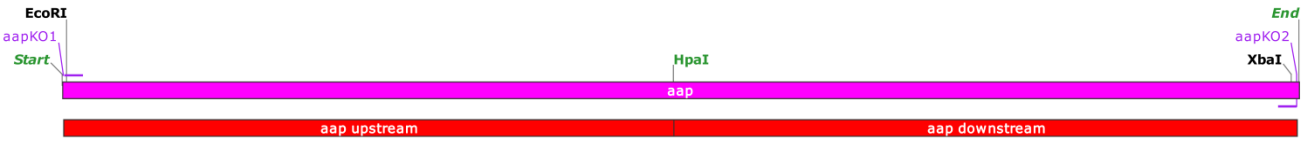
(b)



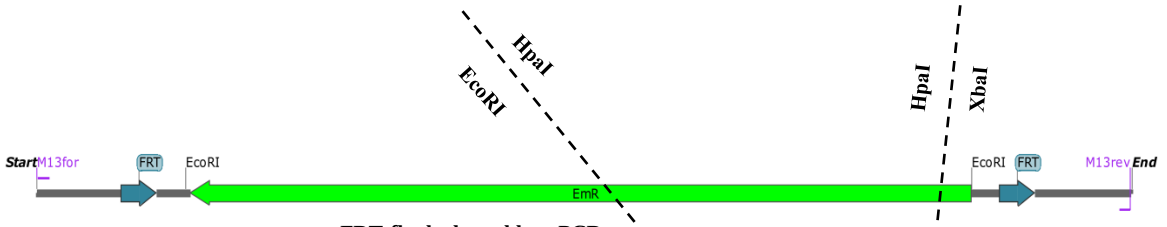
Additional file 1: Fig. S11. Gene deletion and integration via homologous recombination into the genome of *E. mundtii* ST4SA at the *srtC* locus to create *E. mundtii* ST4SA *srtC*::FRT*erm*. **(a)** Schematic representing the wild-type (WT) *E. mundtii* ST4SA *srtC* gene locus and the recombinant *srtC* deletion and FRT-*erm* integration site. Boxed regions show the upstream (~ 1 kb) and downstream (~ 0.4 kb) regions of homology on the WT chromosome and the recombinant *srtC*::FRT*erm* locus. Cells harboring the *srtC* KO vector were selected on Cm and Em, followed by nisin induction for MazF toxin expression to select for mutants that have lost the plasmid backbone bearing *cat* and *mazF* genes. Double crossover mutants were selected and screened by PCR using the indicated primer combinations. **(b)** PCR amplification of WT (3142 bp) and *srtC* deletion and insertion mutants (3183 bp) using the primer pairs indicated in panel B, followed by restriction digestion of the amplicons with XbaI. The WT amplicon contained one *Xba*I restriction site, while the *srtC* mutant amplicons contained three *Xba*I restriction sites. **(m)** Lambda DNA digested with *Pst*I (NEB). Digested amplicons from one WT and three *srtC*::FRT*erm* insertion mutant colonies are shown.



Additional file 1: Fig. S12. (a) Schematic representing the construction of the MazF toxin expression plasmids pNZmazF, pNZmazFnisRK and (b) the arrangement of PnisA promoter placed upstream of the *mazF* gene. Relevant restriction sites are shown. Construction of the plasmid is best understood by referring to "Materials and Methods" in the main text.

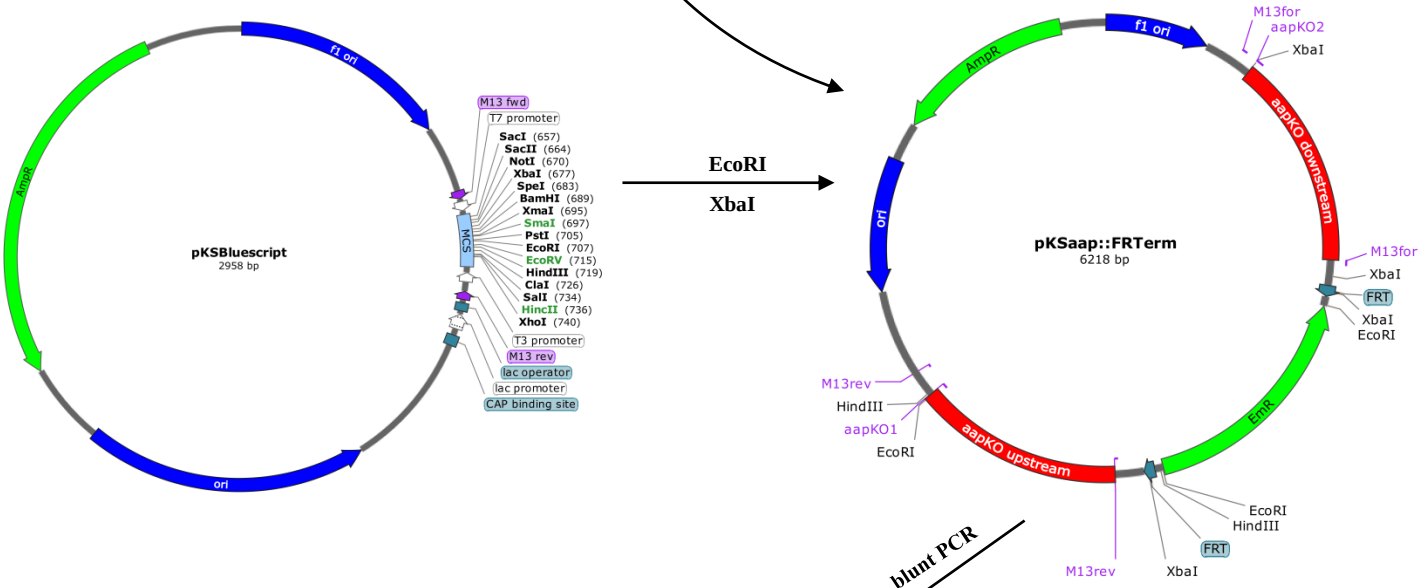


Amplicon size: ~ 1.9 kb

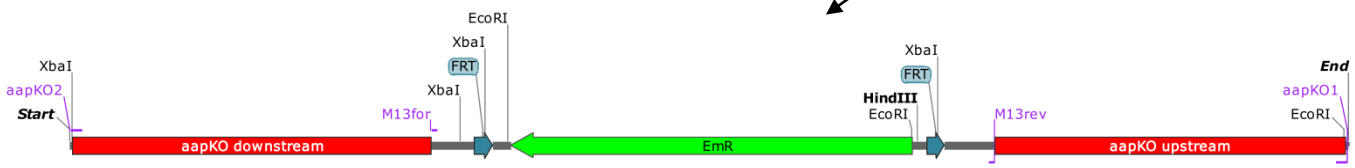


FRT-flanked *erm* blunt PCR

Amplicon size: ~ 1.5 kb

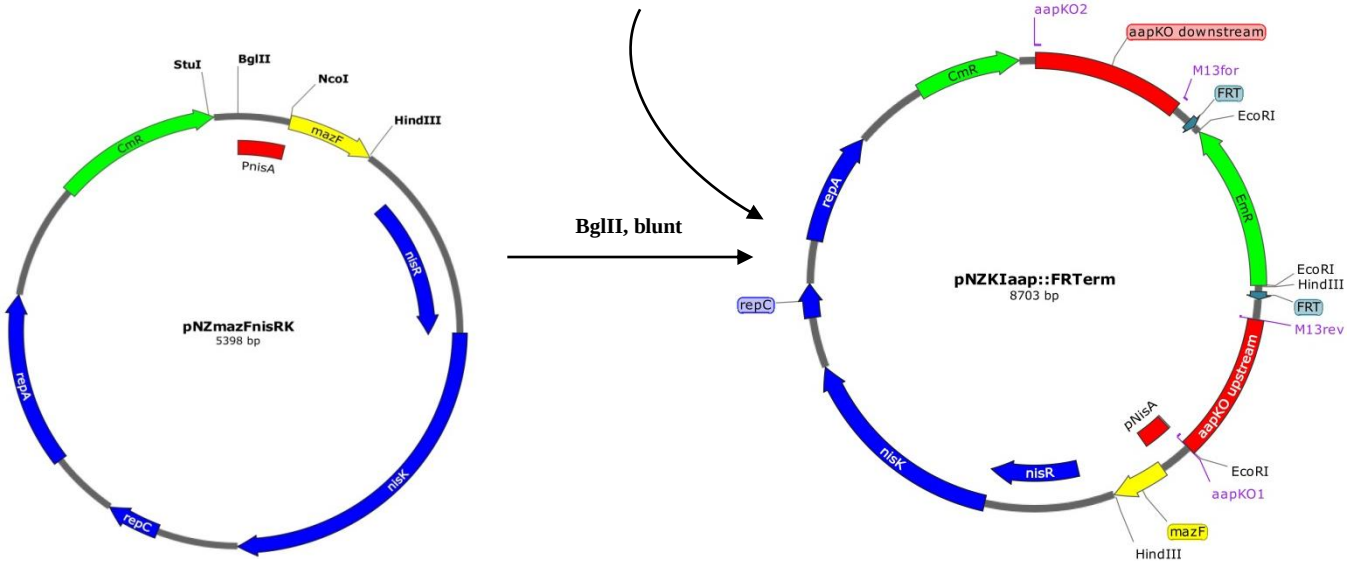


blunt PCR

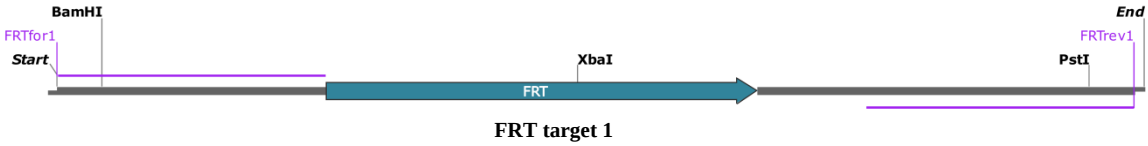


***aap*::FRT*erm* amplicon**

Amplicon size: ~ 3.3 kb

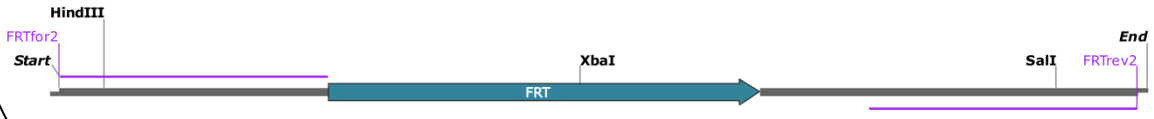


Additional file 1: Fig. S13. Schematic representing the construction of the pNZKIaap::FRTerm integrative plasmid containing the *erm* gene and two flanking sequences of the *aap* mucus adhesion gene from *L. plantarum* 423. Relevant genes, restriction sites and PCR primers are shown. Construction of the plasmid is best understood by referring to "Materials and Methods" in the main text.



FRT target 1

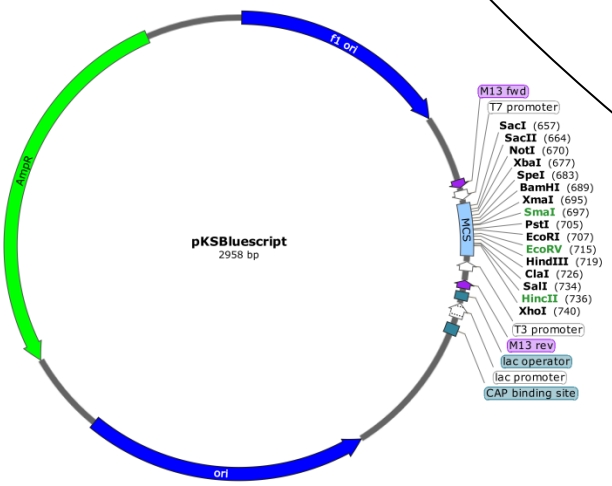
Amplicon size: 121 bp

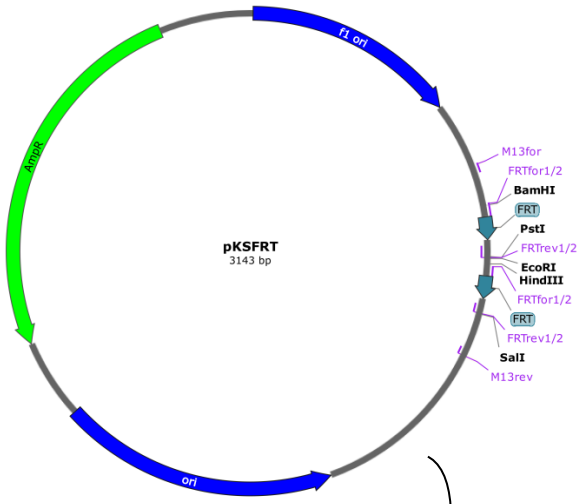


FRT target 2

Amplicon size: 121 bp

BamHI **PstI** **HindIII** **SalI**

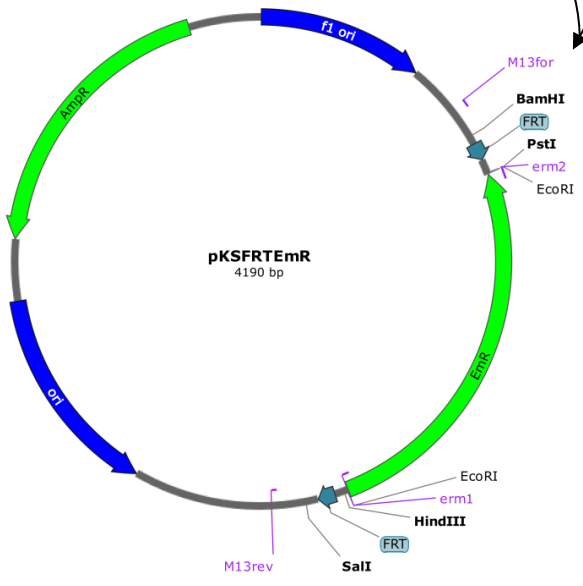




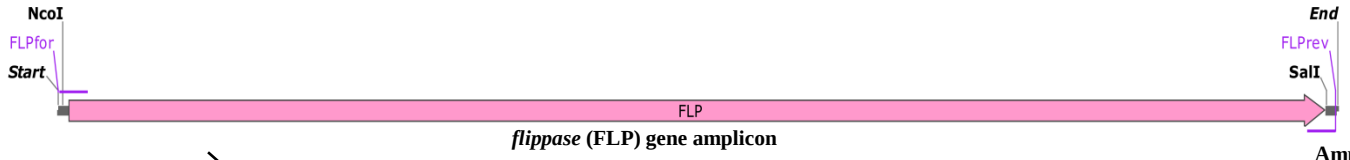
Amplicon size: ~ 1 kb

***erm* gene amplicon**

EcoRI **EcoRI**



Additional file 1: Fig. S14. Schematic representing the construction of plasmid pKSFRTErm containing the FRT-flanked *erm* gene. Relevant genes, restriction sites and PCR primers are shown. Construction of the plasmid is best understood by referring to “Materials and Methods” in the main text.

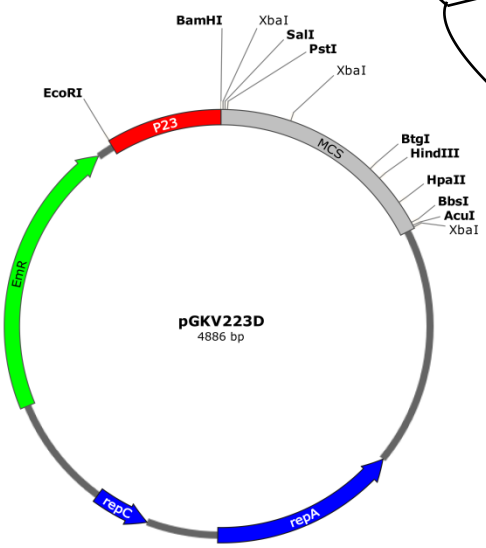


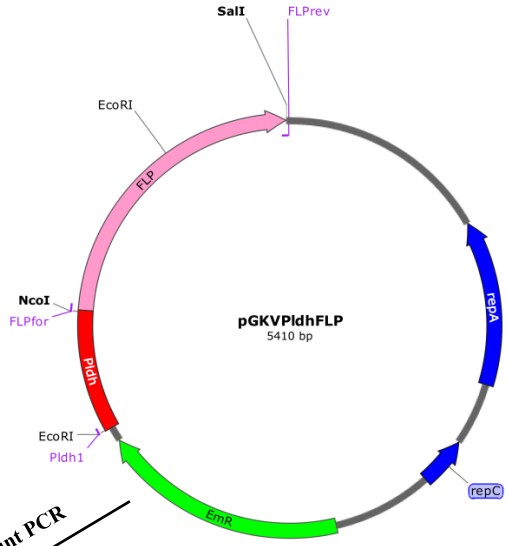
flippase (FLP) gene amplicon

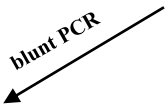
Amplicon size: ~ 1.3 kb

Pldh constitutive promoter

Amplicon size: ~ 0.5 kb

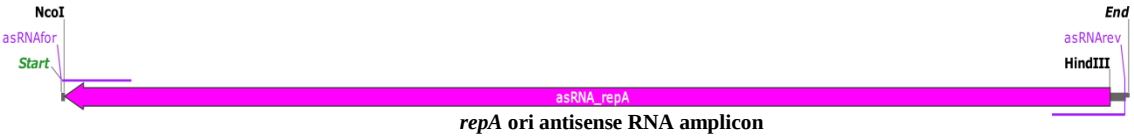






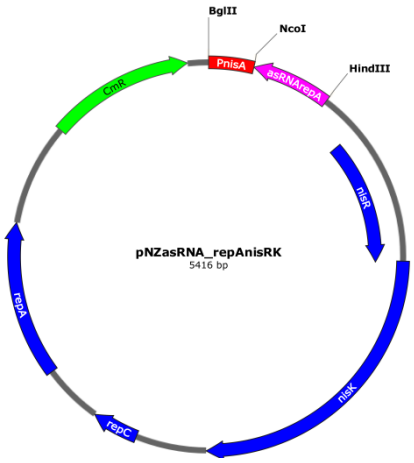
Pldh*flp* amplicon

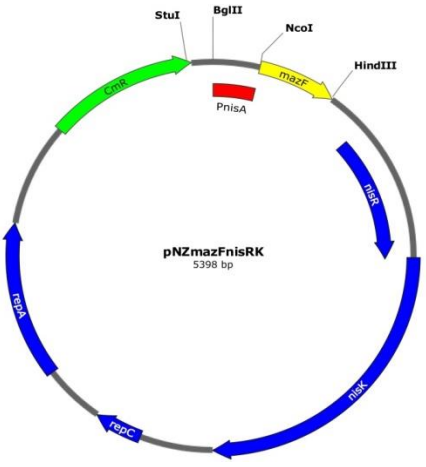
Amplicon size: ~ 1.8 kb

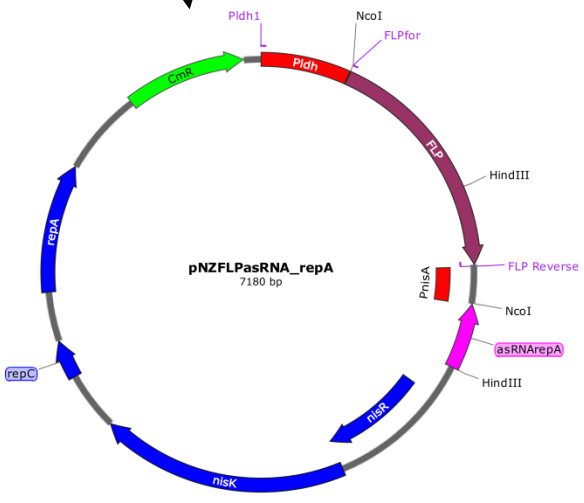


repA ori antisense RNA amplicon

Amplicon size: ~ 3.5 kb







Additional file 1: Fig. S15. Schematic representing the construction of plasmid pNZFLPasRNA_repA containing the *flp* recombinase gene and a *repA* asRNA fragment. Relevant genes, restriction sites and PCR primers are shown. Construction of the plasmid is best understood by referring to "Materials and Methods" in the main text.