

**Table S1:** oligonucleotides used in this study.

Primer designation	Sequence (5' to 3')	Purpose of use	PCR conditions <sup>a</sup>
pseudo_R pcp_R2	TCATTAATCACCCCTTTTCC GTCCGCTCCATACGATACCG	Confirmation of a new junction, obtained after inversion <i>via</i> 197-bp IRs (Fig. 1B). The PCR product is 824 bp.	T=58° C; t=45 sec
IS2010-0923-R antisig_R1	ACAGTCTTTGATATCCTAATG CTCCAAATCATAGAGCCACC	Confirmation of a new junction, obtained after inversion <i>via</i> 197-bp IRs (Fig. 1B). The PCR product is 1,979 bp.	T=56° C; t=2 min
aadE_R3 <sup>b</sup>	ATCATCATACTCCCTTGCGC	Confirmation of a new junction, obtained after inversion <i>via</i> 155-bp IRs (Fig. 1C); used with IS2010-0923-R. The PCR product is 1,066 bp.	T=54° C; t=45 sec
CPT_R pinE1_F1	GCCACGGCTCTAAAAACCAC AACAGCCACGGTTAACAGAG	Confirmation of a new junction, obtained after inversion <i>via</i> 155-bp IRs (Fig. 1C). The PCR product is 638 bp.	T=60° C; t=45 sec
0922_down_F1 up-aadE_F2	GCGTACAGTTAAACTCACAG GCACATTCGGTAACGGAAGC	Restored sequence junction after deletion (excision) of the <i>aadE</i> *- <i>sat4</i> - <i>aphA</i> -3 gene cluster <i>via</i> 155-bp DRs (WT; Fig. 1A) as well as after excision <i>via</i> 197-bp DRs (Fig. 1C). The PCR product is 727 bp. In addition, these primers were used in semi-quantitative PCR to test excision of the <i>aadE</i> *- <i>sat4</i> - <i>aphA</i> -3 cluster during nonselective serial passages (Fig. S6A).	T=56° C; t=30 sec
0926_197_DR_R1	ACAAGAGTGGTGCGCTTAC	Used with aadE_R3 to detect a circularization of the <i>aadE</i> *- <i>sat4</i> - <i>aphA</i> -3 genes-containing region occurred <i>via</i> 155-bp DRs (WT; Fig. 1A) as well as <i>via</i> 197-bp DRs (Fig. 1C). The PCR product is 2,921 bp.	T=59° C; t=1 min 45 sec
tRNA_Leu_F	AGTGGTGAAATTGGCAGAC	Used with pseudo_R to detect excision of the prophage during multiple	T=59° C; t=15 sec

		passages under non-selective conditions (Fig. S6B). In the prophageless strains obtained PCR product is of 378 bp.	
IS1_del_For	<b>GCGGAGTGTATACTGGCTTAC</b>	Amplification of the pACYC184 derivative plasmid without <i>tet</i> gene as well as P2 and P4 promoters (pACYC_Δtet ΔP2 <sup>vec</sup> ΔP4 <sup>vec</sup> ). First step PCRs: IS1_del_For was used with NEW_HindIII_pACYC184, while IS1_del_Rev was used with pACYC184-R1-SphI. Second step assembly PCR was performed with NEW_HindIII_pACYC184 and pACYC184-R1-SphI using PCR products obtained in the first step.	First PCRs (both reactions): T=58° C; t=1min 30 sec. Second PCR: T=60° C; t=2min
NEW_HindIII_pACYC184	atta <b>AAGCTT</b> ACATGAGAATTACAAC		
IS1_del_Rev	<b>GTAAGCCAGTATACACTCCGC</b>		
	CGACGCACTTTGCGCCGAA		
pACYC184-R1-SphI <sup>b</sup>	tata <b>G</b> CATGCGTATTAACGAAGCGCTAACC		
woP2_aphA_SphI	atta <b>G</b> CATGCTGTAGAAAAGAGGAAG	Amplification of the P2-promoterless <i>aphA-3</i> gene to clone into pACYC_Δtet ΔP2 <sup>vec</sup> ΔP4 <sup>vec</sup> in the same direction as <i>cat</i> gene (pACYC_ΔP2_aphA3-17). The PCR product is 855 bp.	T=56° C; t=45 sec
aphA3-R1-HindIII <sup>b</sup>	atta <b>AAGCTT</b> TTTAGATATCTAAATCTAGG		
woP2_aphA_HindIII	atta <b>AAGCTT</b> TGTAGAAAAGAGGAAG	Amplification of the P2-promoterless <i>aphA-3</i> gene to clone into pACYC_Δtet ΔP2 <sup>vec</sup> ΔP4 <sup>vec</sup> in direction opposite of <i>cat</i> gene (pACYC_ΔP2_aphA3-19). The PCR product is 855 bp.	T=56° C; t=45 sec
down_aphA-R3_SphI	atta <b>G</b> CATGCTTTAGATATCTAAATCTAGG		
up_aadE-F3_HindIII	atta <b>AAGCTT</b> GCACATTCGGTAACGGAAGC	Amplification of the <i>aadE*-sat4-aphA-3</i> gene cluster located under P* promoter to clone into pACYC_Δtet ΔP2 <sup>vec</sup> ΔP4 <sup>vec</sup> in direction opposite of <i>cat</i> gene (pACYC_P*); used with down_aphA-R3_SphI. The PCR product is 2,227 bp.	T=56° C; t=2 min

aadE-F3-HindIII	tataAAGCTTGGACAAGATTGAAAAGTGG	Amplification of the <i>aadE</i> *- <i>sat4</i> - <i>aphA</i> -3 gene cluster under P* <sup>Der</sup> promoter, obtained after inversion through 155-bp IRs (Fig. 1C) to clone into pACYC_Δtet ΔP2 <sup>vec</sup> ΔP4 <sup>vec</sup> in direction opposite of <i>cat</i> gene (pACYC_P* <sup>Der</sup> ); used with down_aphA-R3_SphI. The PCR product is 2,178 bp;	T=56° C; t=2 min
P2_For1_HindIII	tataAAGCTTGGAACAGTGAATTGGAG	Amplification of the <i>aphA</i> -3 gene under P2 promoter to clone into pACYC_Δtet ΔP2 <sup>vec</sup> ΔP4 <sup>vec</sup> in direction opposite of <i>cat</i> gene (pACYC_P2_aphA3); used with down_aphA-R3_SphI. The PCR product is 914 bp.	T=56° C; t=1 min
aadE_del_5'Rev	CTGTTCCCGCCTCTCTTCTATTGTCCCC GGCGCACCTGTG	Amplification of the <i>sat4</i> - <i>aphA</i> -3 genes to clone into pACYC_Δtet ΔP2 <sup>vec</sup> ΔP4 <sup>vec</sup> in direction opposite of <i>cat</i> gene; in this construct the <i>aadE</i> * gene was deleted (pACYC_P* Δ <i>aadE</i> *). First step PCRs: <i>aadE</i> _del_5'Rev was used with up_aadE-F3_HindIII, while <i>aadE</i> _del_3'For was used with down_aphA-R3_SphI. Second step of assembly PCR was performed with up_aadE-F3_HindIII and down_aphA-R3_Sph using PCR products obtained in the first step. The PCR product is 1,675 bp.	First PCRs: T=60° C; t=15 sec or t=1 min 30 sec, respectively. Second PCR: T=60° C; t=1 min 30 sec.
aadE_del_3'For	ATAGAAGAGAGGCGGGAACAGTG		
sat4_F3_Hind	tataAAGCTTACCGAGGTATGAAAACGAG	Amplification of the <i>aphA</i> -3 gene under P1" with deletion of most of the <i>sat4</i> gene as well as P2 promoter of the <i>aphA</i> -3 gene (pACYC_P1" ΔP2_aphA3). First step PCRs: sat4_F3_Hind was used with	First PCRs: T=58° C; t=15 sec or t=45 sec, respectively. Second PCR: T=60° C; t=1 min.
sat4-R2 <sup>b</sup>	TATAAGCGTACCGGTTCC		
aph_Pdel_For	AATTGGAACCGGTACGCTTATACTGTA GAAAAGAGGAAGG		

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sat4-R2, while aph\_Pdel\_For was used with down\_aphA-R3\_SphI. Second step of assembly PCR was performed with sat4\_F3\_Hind and down\_aphA-R3\_SphI using PCR products obtained in the first step. The PCR product is 1,071 bp.

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<sup>a</sup> T – annealing temperature; t – amplification time; number of amplification cycles were either 30 or 35.

<sup>b</sup> Sequence of these primers have been previously published [1].

*Hind*III and *Sph*I sites are underlined, while 5' nucleotides, added to improve restriction cleavage, are shown as lowercase letters.

Complementary sequences present in the primers used in the assembly PCRs are bolded.

When needed, internal primers were used to confirm the sequences obtained (data not shown).

The size of the *aadE*\*-*sat4*-*aphA*-3 gene-related PCR constructs includes *Hind*III and *Sph*I sites.

## References

1. Lysnyansky, I.; Borovok, I. A GC-rich prophage-like genomic region of *Mycoplasma bovirhinis* HAZ141\_2 carries a gene cluster encoding resistance kanamycin and neomycin. *Antimicrob Agents Chemother* **2021**, *65*:e01010-20. doi: 10.1128/AAC.01010-20.

**Table S2:** description of the recombinant plasmids used in this study.

Plasmid construct <sup>1</sup>	Description of the construct
pACYC $\Delta$ tet $\Delta$ P2 <sup>vec</sup> $\Delta$ P4 <sup>vec</sup>	Derivate of pACYC184 plasmid without <i>tet</i> gene as well as P2 and P4 promoters (Fig. S4).
pACYC_ $\Delta$ P2_aphA3-17	P2-promoterless <i>aphA-3</i> gene (Fig. S5) cloned into pACYC_ $\Delta$ tet $\Delta$ P2 <sup>vec</sup> $\Delta$ P4 <sup>vec</sup> plasmid in direction of the <i>cat</i> gene.
pACYC $\Delta$ P2_aphA3-19	P2-promoterless <i>aphA-3</i> gene (Fig. S5).
pACYC_P*	The <i>aadE</i> *- <i>sat4</i> - <i>aphA-3</i> gene cluster is under P* promoter, located upstream of <i>aadE</i> * gene (Fig. 1A and Fig. S5).
pACYC_P* <sup>Der</sup>	The <i>aadE</i> *- <i>sat4</i> - <i>aphA-3</i> gene cluster is under derivate (Der) P* promoter, obtained after inversion through 155-bp IRs (Fig. 1C, Fig. 2, and Fig. S5).
pACYC P* $\Delta$ <i>aadE</i> *	The <i>sat4</i> - <i>aphA-3</i> genes are under P* promoter; the <i>aadE</i> * gene is deleted (Fig. S5).
pACYC_P1" $\Delta$ P2_aphA3	The <i>aphA-3</i> gene is under P1" promoter, located within the <i>sat4</i> gene. In this construct the P*, <i>aadE</i> * and P2 sequences were deleted. In addition, in this construct 5'- as well as 3'-encoding sequences of <i>sat4</i> gene were deleted remaining mostly the P1"- <i>sat4</i> containing region (Fig. S5).
pACYC_P2_aphA3	The <i>aphA-3</i> gene is under P2 promoter (Fig. S5).

<sup>1</sup> All constructs were cloned into pACYC\_ $\Delta$ tet $\Delta$ P2<sup>vec</sup> $\Delta$ P4<sup>vec</sup> derivate in orientation opposite of the *cat* gene, otherwise indicated.