Dual control of flagellar synthesis and exopolysaccharide production by FlbD-FliX class-II regulatory proteins in *Bradyrhizobium diazoefficiens*

Supplementary Material

Carolina Dardis[1,&], J. Ignacio Quelas[1,#], Florencia Mengucci[1], M. Julia Althabegoiti[1], Aníbal R. Lodeiro[1,2], Elías J. Mongiardini[1,*]

[1]Instituto de Biotecnología y Biología Molecular (IBBM). Facultad de Ciencias Exactas, Universidad Nacional de La Plata y CCT-La Plata, CONICET. Calles 47 y 115 (1900) La Plata, Argentina

[2]Cátedra de Genética. Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata. Calles 60 y 119 (1900) La Plata, Argentina

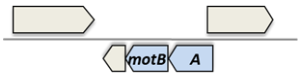
[#]Present address: Y-TEC, Avenida del Petróleo Argentino e/129 y 143 (1923) Berisso, Argentina

[&]Present address: Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, UNLP-CIC-CONICET), La Plata, Argentina

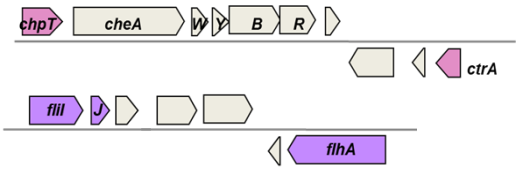
*Corresponding author. e-mail: mongiardini@biol.unlp.edu.ar

FIG. S1

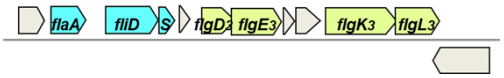
Cluster 1 (from bll1510 (1,645,098 bp) to bll1511 (1,647,162 bp))



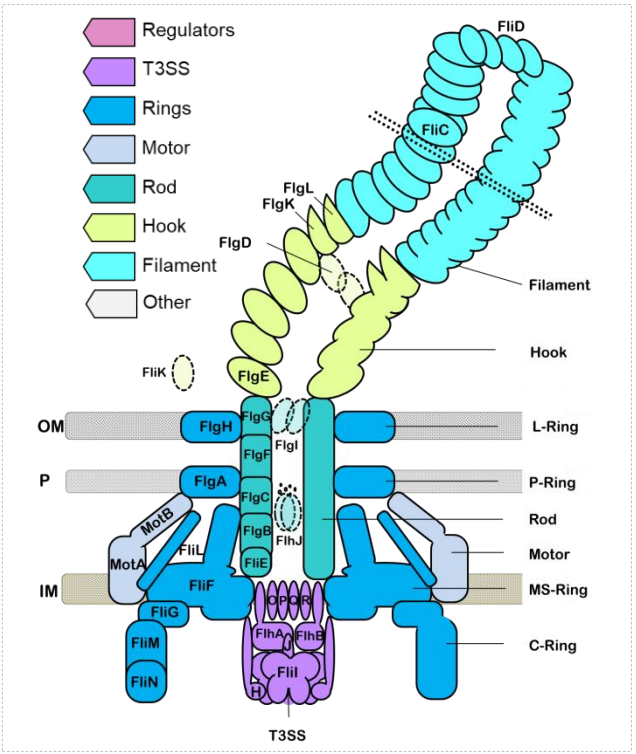
Cluster 2 (from blr2191 (2,366,175 bp) to bll2207 (2,386,548 bp))



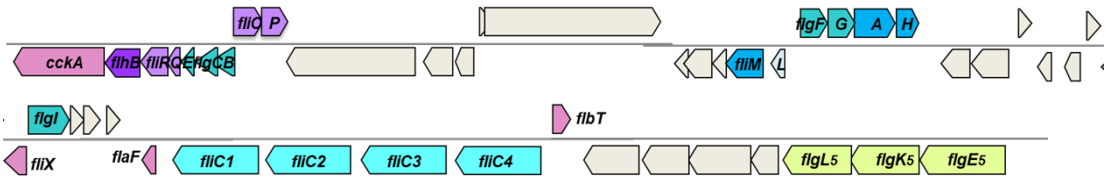
Cluster 3 (from blr3695 (4,085,820 bp) to blr3704 (4,094,903 bp))



Cluster 4 (from bll3800 (4,211,935 bp) to bll3801 (4,213,563 bp))



Cluster 5 (from bll5808 (6,372,634 bp) to bll5854 (6,431,041 bp))



Cluster 6 (from blr6996 (7,704,420 bp) to blr7003 (7,712,770 bp))

FIG S1. Scheme of the six gene clusters related to the subpolar flagellum system of *B. diazoefficiens* USDA 110. The genes assigned to each cluster are indicated between parentheses, including the locus tag and the start and end positions in bp. The genes were named by function similarity and colored according to the putative structural or regulatory function show in the scheme.

FIG. S2

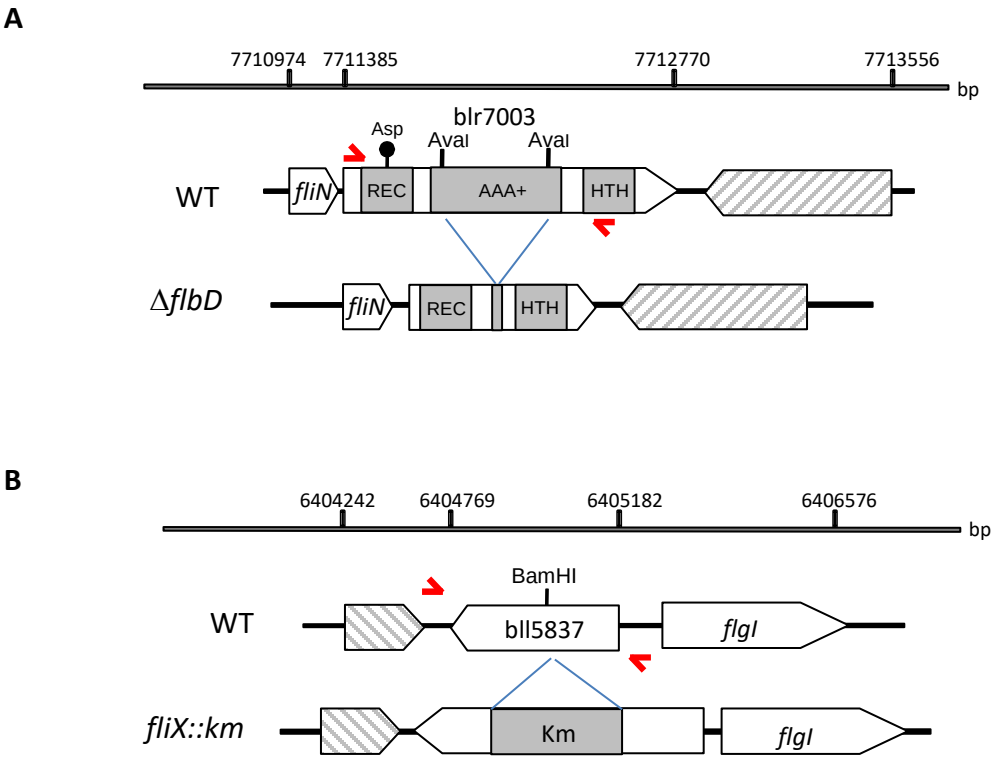


FIG S2. Scheme of the mutagenesis strategy for class-II regulators. Primers are represented with red arrows and the deletion or insertion events are represented with blue lines. The stripped boxes indicate no flagellar genes. (A) Deletion strategy for *flbD* (blr7003) mutation. The three characteristic domains of FlbD are represented with grey boxes and both AvaI restriction sites used for deletion are shown in the WT scheme. (B) Insertion strategy for *fliX* (bll5837) mutation. The BamHI restriction site is shown in the WT scheme. In this case, the grey box represents the Km-resistant gene.

FIG. S3

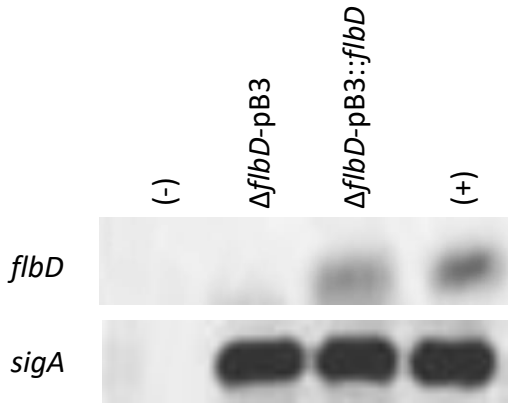
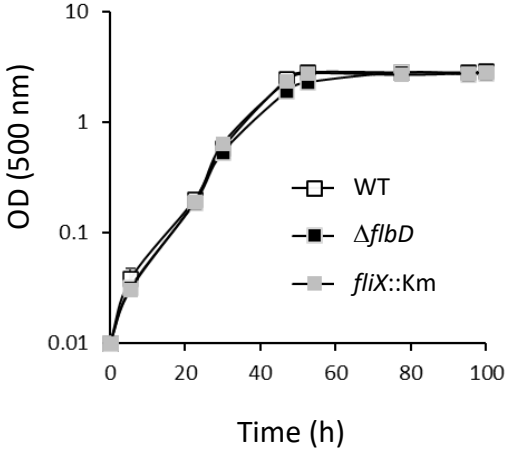


FIG S3. Complementation assay of the FlbD mutant. RT-PCR of the *flbD* mutant and the mutant complemented with the wild-type gene in replicative plasmid pB3. Positive (+) and negative (-) PCR controls were performed using DNA and purified RNA as templates, respectively. *sigA* was used as endogenous control gene.

FIG. S4

A



B

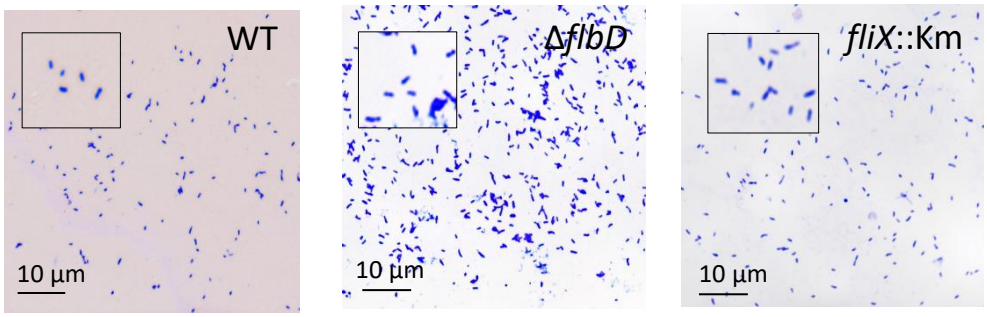


FIG S4. Growth of Δ*flbD* and *fliX*::Km mutants. (A) Growth kinetics of the different strains. The graphic represents the mean ± SEM of two independent experiments (error bars not shown are smaller than the symbols). (B) Light micrographs of bacteria grown in PSY-Ara exponential phase and stained with crystal violet. The boxes in each photograph show a part of the same picture at 400 X magnification.

FIG. S5

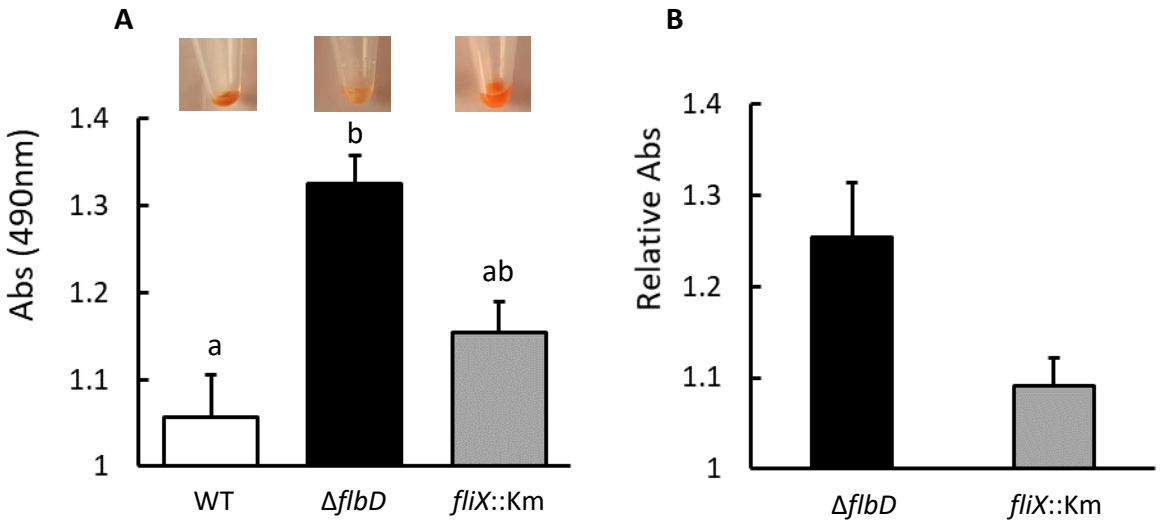


FIG S5. Congo red (CR) binding assay. (A) Quantification of remaining CR present in the supernatant of each strain grown in PSY-Ara. Different letters indicate statistically significant differences (ANOVA, $p<0.05$). Photographs above the bars are representative of the remaining pellets of each supernatant measured. (B) Relative absorbance calculated according to Material and methods. The bars represent the standard deviation.

FIG. S6

```
C. crescentus NA1000                MRLLVVGKLNGQLSVAVKMAMNAGAKVSHVETTEQATNALRAGQGADLLMVDYTLDIAGM 60
B. sachari BR10555                  MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. huanghuaihaiense CGMCC1.10948    MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. yuanmingense CGMCC1.3531        MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. manausense BR3351                MRLLIVGTLKGQLTTATKIAMDNGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. ottawaense L2                    MRLLIVGTLKGQLTTATKIAMDNGATVTHAEDHDQAMRVLRGGKGADLLLVDVALDIRDL 60
B. japonicum USDA6                  MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. japonicum E109                   MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. lupini DSM30140                  MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. japonicum NK6                    MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. japonicum USDA110                MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. japonicum USDA122                MRLLIVGTLKGQLTTATKIAMENGATVTHAEDHEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. betae CECT5829                   MRLLIVGTLKGQLTTATKIAMENGATVTHAEDLEQAMRVLRGGKGADLLLVDVALDIRDL 60
B. canariense UBMA181               MRLLIVGTLKGQLTTATKIAMANGATVTHAEDNEQAMRVLRGGKGADLLLVDVALDIRDL 60
                                    ****:**.*:***:.*.*:**  **.*:*.*  :** ..**.*:*****:** :*** .:
```

FIG S6. Amino acid sequence alignment of FlbD homologs. Alignment of the N-terminal sequence (first 60 aa) of FlbD of different *Bradyrhizobium* species, and *C. crescentus*. Highlighted in bold is the conserved aspartate-52 residue involved in post-translational modification in *C. crescentus* (1). Aspartate residues at positions 47 and 56 also are conserved.

Table S1. Strains and plasmids used in this study.

Strains or plasmids	***Relevant genotype or phenotype***	***Source***
B. diazoefficiens		
WT	Wild-type strain USDA 110, Cm^R	ARS-USDA, Beltsville (MD)
Δ*flbD*	WT derivative, *flbD* deletion mutant	This study
fliX::Km	WT derivative, *fliX* mutant by Km insertion	This study
Δ*flbD-fliX::*Km	Δ*flbD* derivative, *fliX* mutant by Km insertion	This study
WT-*Spc*4	USDA 110 derivative, Spc^R	(2)
∆*rpoN1*	*Spc4* derivative, *rpoN1* mutant by Km insertion	(3)
∆*rpoN2*	*Spc4* derivative, *rpoN2* mutant by Km insertion	(3)
∆*rpoN1*-2	∆*rpoN1* derivative, *rpoN2* mutant by Sm insertion	(3)
Δ*fliC*	WT derivative, *fliC1-2-3-4* deletion mutant and Km insertion	(4)
flbD-D52A	Δ*flbD* derivative, point mutation in the residue D52A	This study
WT-pB3	WT carrying the pBBR1MCS3 plasmid	This study
WT-pB3::*flbD*	WT carrying the pB3::*flbD* plasmid	This study
Δ*flbD*-pB3	Δ*flbD* carrying the pBBR1MCS3 plasmid	This study
Δ*flbD*-pB3::*flb*D	Δ*flbD* carrying the pB3::*flbD* plasmid	This study
WT-pFAJ	WT carrying the pFAJ1708 plasmid	This study
WT-pFAJ::*fliX*	WT carrying the pFAJ::*fliX* plasmid	This study
fliX::Km-pFAJ::*fli*X	*fliX*::Km carrying the pFAJ::*fliX* plasmid	This study
E.coli		
DH5α	F– *endA1 supE44 thi-1l-recA1 gyrA96 relA1 deoRD*(*lacZYA-argF*)U169	Bethesda Research Laboratory
S17-1	*E. coli* 294 Thi RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	Bethesda Research Laboratory
Plasmids		
pG18mob2	*lacZ*α Mob^+ Gm^R, suicide vector in rhizobia	(5)
pK18mob*sacB*	*lacZ*α Mob^+ Km^R *sacB,* suicide vector in rhizobia	(6)
pUC4K	Plasmid with *nptI* gene (source for Km-resistance cassette) Ap^R, Km^R	(7)
pBlueScriptSK(+)	*lacZ*α Ap^R	Stratagene, La Jolla, CA, USA
pBBR1MSC3	*lacZ*α broad-host-range expression vector containing p*lac* promoter, Tc^R	(8)
pFAJ1708	broad-host-range expression vector containing P*nptII* promoter, Ap^R,Tc^R	(9)
pK*sacB*::*flbD*	pK18mob*sacB* carrying the *flbD* gene	This study
pK*sacB*::Δ*flbD*	pK*sacB::flbD* digested with *AvaI* and religated	This study
pKsacB::flbD-D52A	pK*sacB::flbD* with a specific mutation in Asp52 amino acid	This study

pB3::*flbD*	pBBR1MSC3 carrying the *flbD* gene	This study
pB::*fliX*	pBlueScriptSK(+) carrying the *fliX* gene	This study
pG::*fliX*	pG18*mob2* carrying the fragment of the *fliX* gene	This study
pG::*fliX*::Km	pG::*fliX* carrying the Km cassette in the middle of the *fliX* gene	This study
pFAJ::*fliX*	pFAJ1708 carrying the *fliX* gene	This study

Table S2. Primers used in this study.

Primer name	Sequence	Use in the study
7003FwE	AAAAgaattcTCAAGACCGAACGCACGTAA	Construction of the *flbD* deletional mutant
7003RvH	AAAAagcttcGGCGTATTCGTTGAGCTTG	
E7003FwX	AAtctagaGGCCATCGACATCTACGTCA	Checking the position of the crossing-over event in the *flbD* mutant and construction of pB3::*flbD*
E7003RvP	AAActgcagACTTATTGTGTGGCGACTGGT	
5837Fw	TATCACGCAACTCGGGCTTAC	Construction of the *fliX* insertional mutant and construction of pFAJ::*fliX*
5837Rv	CCCAACAATCCTCACCCAAC	
E5837Fw	ACAAATGGGCGGTGCTGA	Checking the position of the crossing-over event in the *fliX* mutant
E5837Rv	CCGCACGCCCTCGATATT	
KmFw	CATCGGGCTTCCCATACA	Checking for Km cassette in the crossing-over event for *fliX* mutation.
KmRv	TGCCATTCTCACCGGATT	
7003D52AFw	CCTCCTGCTGGTCG<u>C</u>CGT	Change of codon #52 GAC (D) to GCC (A) in *flbD* coding gene
7003D52ARv	ACG<u>G</u>CGACCAGCAGGAGG	
q2201Fw	CAAGTGATGGCGACCTATGC	Measurement of mRNA by RT-PCR or qRT-PCR
q2201Rv	GGTAACCGTCCGCCAATGA	
q2207Fw	GCCCTGATGAAGCAGTTCTC	
q2207Rv	GGAAGGGAATGGTCGGAATG	
q3696Fw	ATATCACGGTGACCAGCACG	
q3696Rv	CCCGTCAGCACCATCTCATA	
q3699Fw	TTCGGTTACTCGCTGTCCTC	
q3699Rv	GGTTGCCGCTGGAGTCCTT	
q3800Fw	GATGAAGAGACCAACCGCAC	
q3800Rv	GATAGGCCAGCAGCATTTCG	
q5808Fw	ATCCAGTCGTGGGTCGTATC	
q5808Rv	TCAGATAGGCCGCATTGGAA	
q5814Fw	TTCGACCAGAACAGGAATGC	
q5814Rv	GCGTAATCCATCTGGTTGCT	
q5816Fw	CCTCCCGCGTAGAGTTCTTT	
q5816Rv	ATTGATGCTGATGTCCTGCG	
q5826Fw	GTCACCGACATCTTCCAGAC	
q5826Rv	CTTGGTCAGCTCTTCCTTGAG	
q5827Fw	CTTCTGATCGGCTTGTCACG	
q5827Rv	AGAAACTCCTCGAACAGCGA	
q5838Fw	GAGGATGGTCCGAGATTTGA	
q5838Rv	CCAACAATCCTCACCCAACG	
q5842Fw	ATCGATGTCCGCCAGAAGAT	
q5842Rv	GGCAGCGATATTGCGATTGA	
q5854Fw	ATCAGCGGCGGTGCGGTCA	
q5854Rv	AGAACAGGTTCCAGGTATCGG	
q6996Fw	ACGTCAGATGTAGCTGCCA	
q6996Rv	TCGCCTGGGTATTACTGTCG	
q6999Fw	GAGCAAGAAGACCGAGGAGACCAA	
q6999Rv	CTCCTTGGTGCGGTCCTGATA	
q7003Fw	GTCAACCTGAAGATCCCACC	
q7003Rv	TTGGCTTCGGCGTACTTCTT	

The enzyme restriction sites added to the primers are shown in lowercase. The underlined base represents the point change for D52A mutation.

References

1. Wingrove JA, Mangan EK, Gober JW. 1993. Spatial and temporal phosphorylation of a transcriptional activator regulates pole-specific gene expression in *Caulobacter*. *Genes Dev* 7:1979-92.
2. Regensburger B, Hennecke H. 1983. RNA polymerase from *Rhizobium japonicum*. *Arch Microbiol* 135:103-9.
3. Kullik I, Fritsche S, Knobel H, Sanjuan J, Hennecke H, Fischer HM. 1991. *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the sigma 54 gene (rpoN). *J Bacteriol* 173:1125-38.
4. Mengucci F, Dardis C, Mongiardini EJ, Althabegoiti MJ, Partridge JD, Kojima S, Homma M, Quelas JI, Lodeiro AR. 2020. Characterization of FliL Proteins *in Bradyrhizobium diazoefficiens*: Lateral FliL Supports Swimming Motility, and Subpolar FliL Modulates the Lateral Flagellar System. *J Bacteriol* 202(5):e00708-19.
5. Kirchner O, Tauch A. 2003. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. *J Biotechnol* 104:287–299.
6. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145: 69-73.
7. Vieira J, Messing J. 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. *Gene* 100:189-94.
8. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, 2nd, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175-6.
9. Dombrecht B, Vanderleyden J, Michiels J. 2001. Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in gram-negative bacteria. *Mol Plant Microbe Interact* 14:426-30.