

Supplementary Material

**InnB, a Novel Type III Effector of *Bradyrhizobium elkanii*
USDA61, Controls Symbiosis With *Vigna* Species**

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1 Supplementary Data

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GCATCATGCCGAGGCTGGGCGTCCAACCTATTTCCAGATCCGCGGTGTGCCCTACAGGGGCGAGTTGGAT
GAAAGCGAGGGGCGCCAGCGCGTTTCGCATCTATCCTGAACGTGGCTGA

Supplementary Data S1. The *innB* gene (accession number KX499541) and its promoter sequence of *Bradyrhizobium elkanii* USDA61. The preceded conserved *tts* box motif is shown in the square box, and the *innB* sequence is shown in bold.

2 Supplementary Figures and Tables

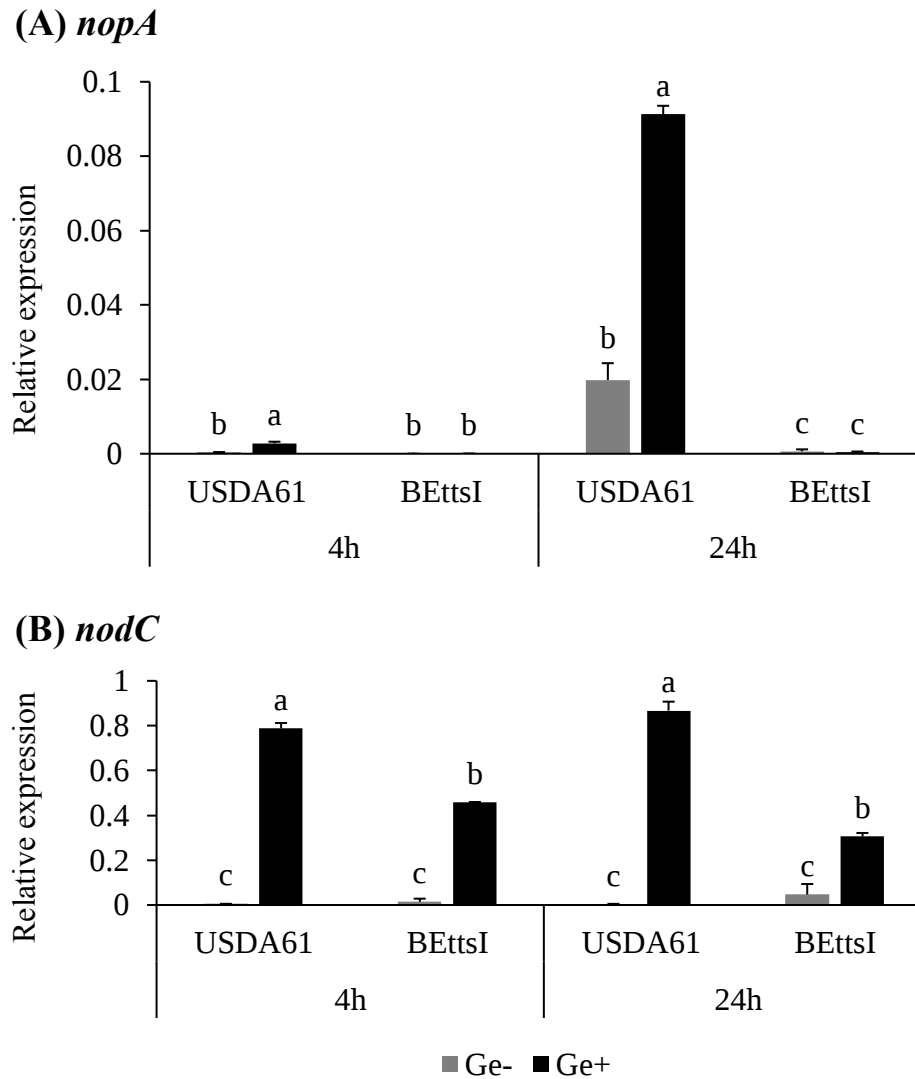
2.1 Supplementary Figures

	1					50
InnB		..MDFVDPFF	DSGAWMEAYA	ARQGRAGQFG	GNASQQGDFF	
SCB50985		MREYA	ALQERTAQRC	DNQERRRGFE		
KRP85897						
BAL13100						
BAC47263						
APO50625		..MDFVDPFF	DSGAWMREYA	ALPGRAGQRR	GDSSEQGDFF	
KGT81182	MLQGLYKHEE	SRMDEVDPFF	DSGAWMREYA	ALPGRAGQRR	GDSSEQGDFF	
	51					100
InnB	RRMEDLQLDS	SDESSAKSSS	PDQAPTGAGR	AVRRDELGGS	MRVPPRSDFR	
SCB50985	RRLEDLSLDP	SDESN..SNS	PDRAPAGGGR	AVRPEDFGGS	MRVPPDSAFR	
KRP85897					MRVPPHSAFG	
BAL13100						
BAC47263						
APO50625	KRMEDLHLDP	SDQSK..STS	PDRPPAGGGR	AVRRDDFGGS	MRAPPHSTFR	
KGT81182	KRMEDLHLDP	SDQSK..STS	PDRPPAGGGR	AVRRDDFGGS	MRAPPHSTFR	
	101					150
InnB	DSARGDARRS	LDIPRFQIDA	PAQVPOSSLR	DDLFSSARYS	LPDAEPGVQA	
SCB50985	ESALGGTRRS	VDIPSFQLPA	SAQVPOSLR	DDPFSSARYS	FPDAESAASA	
KRP85897	ESALDGARRS	ADIPTFHLPA	SAQVPOSSLR	DDLFSSARYS	FPDAEPAAVA	
BAL13100						
BAC47263						
APO50625	ESALGDTRRS	VDIPSFQLTA	ATQVPOSSLR	DDLFSSAR..		
KGT81182	ESALGDTRRS	VDIPSFQLTA	ATQVPOSSLR	DDLFSSAR..		
	151					200
InnB	RSGKSRGLWS	RFKSGVGKAF	GGSS..KSSR	ETAQSDASST	SFRVDYARQP	
SCB50985	KSGKSGGLWS	RFKSGIGKAF	GGSSSEKYSR	NAGQGDAFST	TLRIDYARQP	
KRP85897	KSGKSRGLWS	RFKSGIGKAL	GGSGSEKSPR	EADQSDVFST	NLRIDYARQP	
BAL13100						
BAC47263						
APO50625						
KGT81182						
	201					250
InnB	GRTRGVDPED	EALIKEFRQR	AVRNRTDGTA	AGNLTDGTIR	NAAADLRILS	
SCB50985	GRTRAVPEED	EVLIRDFRNK	A.....	AGNLTDGTIK	NAAADLRHLS	
KRP85897	GRTRGVPAAD	EELIEDFRNK	A.....	AGNLSDGTIK	NAAADLRHLS	
BAL13100						
BAC47263						
APO50625						
KGT81182						

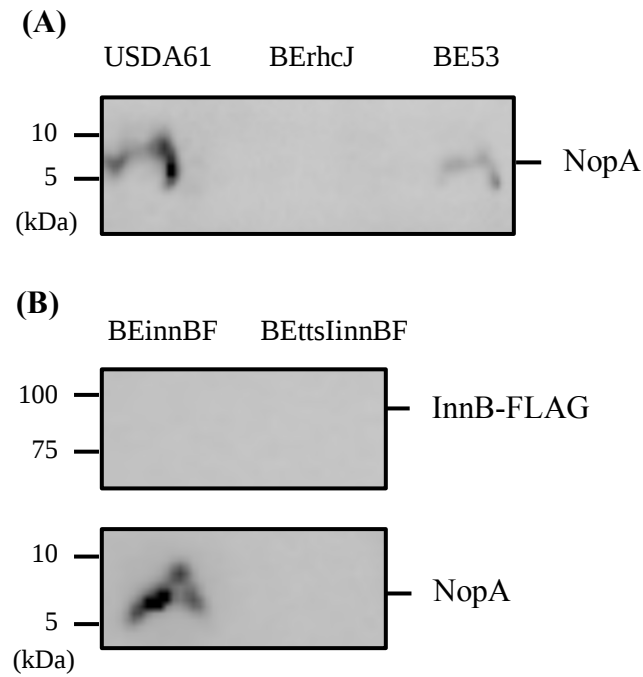
	251				300
InnB	ARLNDNDRPS	IADIRIRLEME	NAQLEDPEVE	NHOLEAELDQ	DVDITYA..AD
SCB50985	ARLSTAGRPS	IADRIQRESE	NAQQDNPEVE	NHOLEAELDE	DVDITYA..KD
KRP85897	ARLSDAGRPS	IADIRIRRELE	NAQLENPDVE	NHOLEAELDQ	DVDAYTHTKD
BAL13100	ME	NAQQDNPEVE	NHOLEAELDE	EVDRYA..GR	
BAC47263	ME	NHOLEAELDE	EVDRYA..GR		
APO50625					
KGT81182					
	301				350
InnB	RRRIKAALK	KLREVGAGNT	LSADLRRRLAP	HSADATLITCM	WAAAEKATRR
SCB50985	RSRRKAALK	KLREVGAGNA	LAPDLRRRLAP	HRADAILITDM	WSAAEKAHR
KRP85897	RGRRIKAALK	KLREVGAGNT	LAA DVRRRLAP	HAEDATLITRM	WAAAEKATGR
BAL13100	RGTRIKAALK	KLREVGAGNA	LAPDLRRRLAP	HGADATLITCM	WAAAEKATRR
BAC47263	RGTRIKAALK	KLREVGAGNA	LAPDLRRRLAP	HGADATLITCM	WAAAEKATHR
APO50625					
KGT81182					
	351				400
InnB	VEPKTIDRQA	RRMSRLSEWL	QKHDKQATAG	RLFTSGLDQD	VAEYRQETED
SCB50985	IEPETIDRQA	RRMYRLSEWL	QTHDRPAMAG	RLSTPALDRD	VEEYRRETKD
KRP85897	IDPKTIDRQA	RRMFRLSEWL	QTHDRLPITAG	RLSTPAFLQD	VEEYRRETD
BAL13100	IEPKTIDRQA	RRMSKLSEWL	RTHDRPPMAG	RLSTPAFVRD	LEEYRRETN
BAC47263	IEPKTIDRQA	RRMSKLSEWL	RTHDRPPMAG	RLSTPAFVRD	LEEYRRETN
APO50625					
KGT81182					
	401				450
InnB	GKIKADLLRL	GRYQOILEAN	RALGLHPAED	AGQVVAEGAR	QAHSPQEVPA
SCB50985	GKIKPDLVKL	GQYEQILEAN	RALGLRPPDD	PGQPSWEAAR	QPHSMOEP
KRP85897	SKINPDLVKL	RQYEQILEAN	RALGFRTPED	PGLESREAR	QPHILOEPP
BAL13100	KKINADLVKL	GQYEQILEAN	RALGLRPPED	PGQPSWEAAR	QPHALOGPP
BAC47263	KKINADLVKL	GQYEQILEAN	RALGLRPPED	PGQPSWEAAR	QPHALOGPP
APO50625					
KGT81182					
	451				500
InnB	TPATPSAGAW	DWLGEQIHGP	TSSLAVPHHG	WQASSSQQLP	ATSASPSAGA
SCB50985	TPATPSAGAW	DWLGEQIHGP	TTS MRAPSD	WQAGSSQQLP	AAPATPSAAA
KRP85897	TPATPSAGAW	DWLGEQIHGP	TTSMPPTPSG	WQAGASQQLP	ATPATPSGA
BAL13100	TPASPSAGAW	DWLGEQIHGP	NISMPAPPSH	WQAGSSQQLP	ATPATPSAGA
BAC47263	TPASPSAGAW	DWLGEQIHGP	NISMPAPPSH	WQAGSSQQLP	ATPATPSAGA
APO50625					
KGT81182					
	501				550
InnB	WDRSSEQILQ	PAPVQAPYW	LPOPNIHPGL	PATPASLSQG	AWDWLGQQMQ
SCB50985	RDWIGEQIHE	PASPVEAPHW	AAQAHPPLEL	PATPATPSQG	AWAWLGQQMQ
KRP85897	WDWLGEQIHE	PASPVEAPHW	APQAHPPLEL	PVTPATPSEG	AWAWLGQQMQ
BAL13100	WNWLGEQIQG	PASPEFAPHW	GAQAHPPLEL	PATPATPSQG	AWAWLGQQMQ
BAC47263	WNWLGEQIQG	PASPEFAPHW	GAQAHPPLEL	PATPATPSQG	AWAWLGQQMQ
APO50625					
KGT81182					
	551				600
InnB	EPASPSSVRP	RSSNIYSGLD	PLVDLDPSTP	HDHDDARSA	PAPEFRRAPS
SCB50985	EPASPSSVRP	RSSNIYGGLD	SFVDLDPPTP	HDLRDDARSA	PAPEFAVTSW
KRP85897	EPGSTSSVRP	RSSNIYAGLD	SFVDLDPPTP	HDLRDDARSA	PAPEFAAASW
BAL13100	DSASPSCVRP	RSSNIYGGLD	SFVDLDPHT	PRJA.....	
BAC47263	DSASPSCVRP	RSSNIYGGLD	SFVDLDPHT	PRJA.....	
APO50625					
KGT81182					

	601				650
InnB	FAGPSGGAQE	LRDIGAVVGS	DWRHGSQAAS	DVLVDVLGNI	NLLPNQFGPS
SCB50985	FAGPPGPAPPE	LRDIGAIVGA	DWRHGSQAAS	DVLIDVLSNI	NLLPNQFGPS
KRP85897	FAGPPGAAPE	LRDIGAIVGA	NWRHGSQAAP	DVLIDVLGNI	DLLPNQFGPS
BAL13100					
BAC47263					
APO50625					
KGT81182					
	651				700
InnB	QFMINGERYS	ATFGPGGRTD	VRLIHHPRPS	YLDEAGPSQP	LHHPPQIVQA
SCB50985	QFAINGEHYS	ATVGPEGPMD	VRLIHHARAG	GINEAGPSQP	LYRPPQIVRA
KRP85897	QFAINGERYS	ATLGPGGRRD	IRLVHHPRAG	RMNEAGPSQP	LHRPAPIVTA
BAL13100					
BAC47263					
APO50625					
KGT81182					
	701				750
InnB	EQPREGLRDL	GYLIRGGWEH	RERFLPPYLV	RVLQGERIMP	QAGRPTYFQI
SCB50985	ARPDEGTVDL	GYLIRGGWEH	RERFLPDYLV	RVLEGQRIMP	EVGRPTYFDI
KRP85897	RQTGEGAVDL	GYLIRGGWEH	RERFLPPFLV	RVLEGERIMP	QPGRPTYFEI
BAL13100					
BAC47263					
APO50625					
KGT81182					
	751		775		
InnB	RGVPYRGELD	ESEGRQVRRI	YPERG		
SCB50985	RGVPYRGELV	ESEGRRRVRA	YPERG		
KRP85897	RGMPYRGELV	ESEGRQVRV	YPEPR		
BAL13100					
BAC47263					
APO50625					
KGT81182					

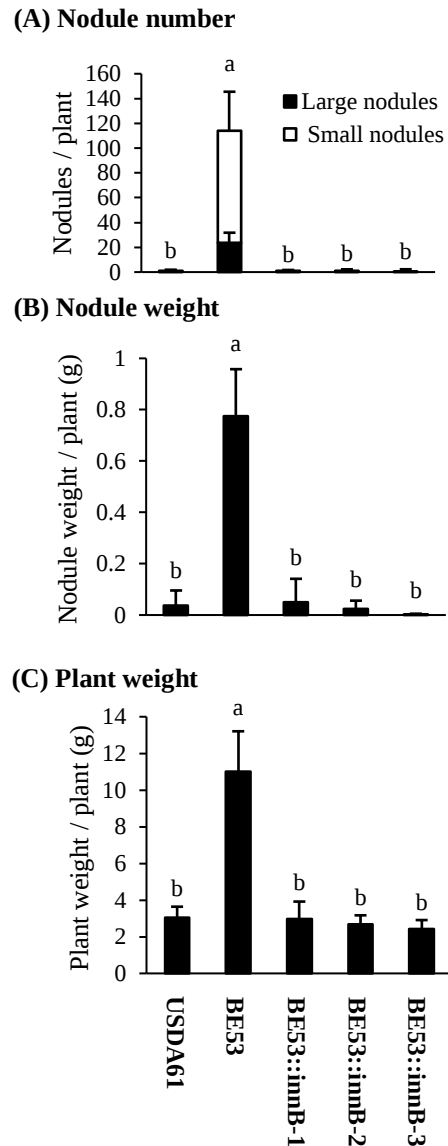
Supplementary Figure S1. Alignment of InnB and its homologs in several rhizobial strains, including *B. yuanmingense* CCBAU10071 (SCB50985, 72% identity), *B. yuanmingense* BR3267 (KRP85897, 73% identity), *B. japonicum* USDA6 (BAL13100, 71% identity), *B. diazoefficiens* USDA110 (BAC47263, 70% identity), *B. diazoefficiens* USDA122 (APO50625, 70% identity) and *B. japonicum* Is-34 (KGT81182, 70% identity). Highly conserved amino acid positions are highlighted in black. The results highlighted by grey indicate the high-conserved amino acids among *B. yuanmingense* strains BR3267 and CCBAU10071.



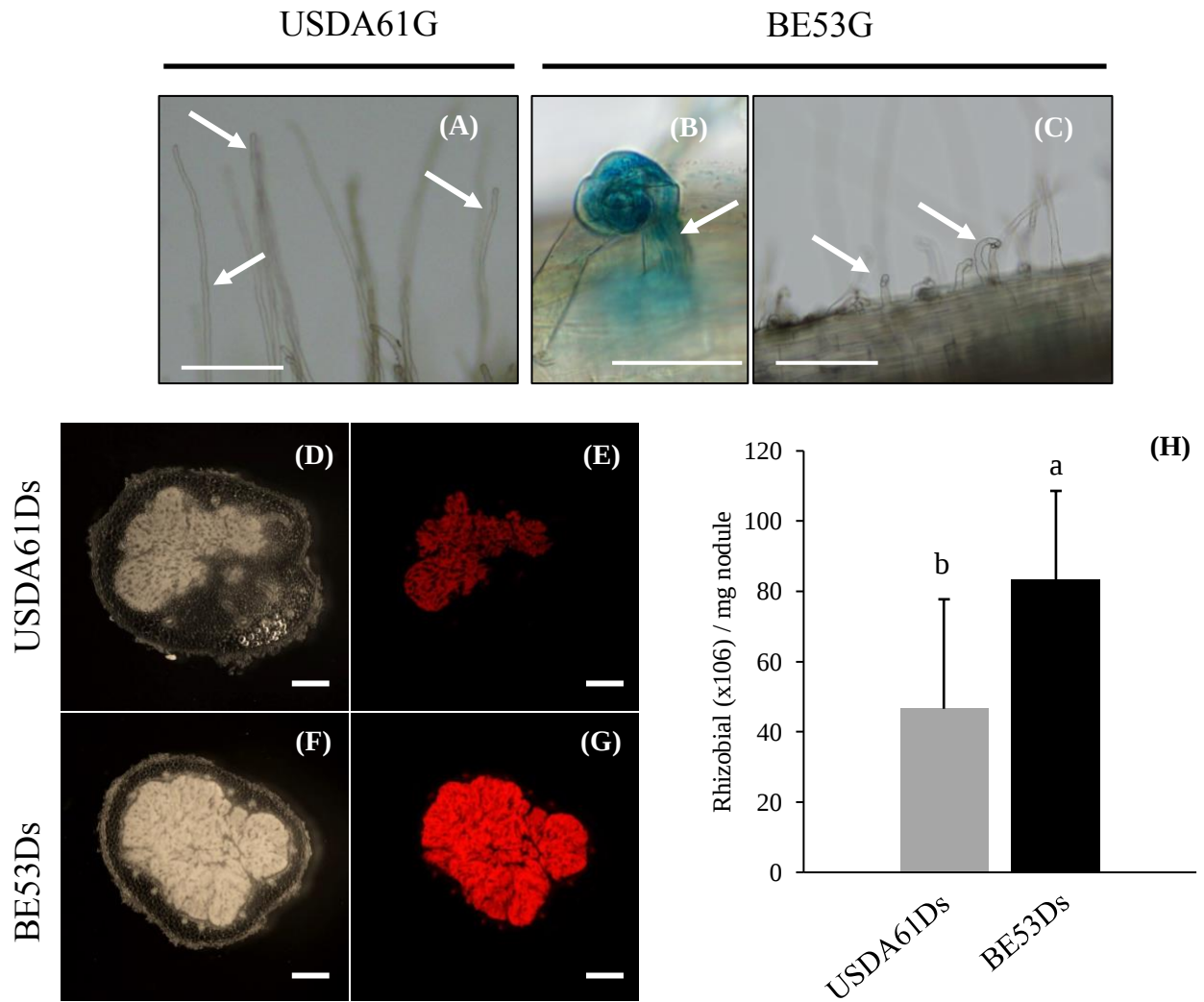
Supplementary Figure S2. Expression of *nopA* and *nodC* in wild-type and mutant *Bradyrhizobium elkanii* strains. Real-time RT-PCR was performed using total RNAs isolated from USDA61 and the *ttsI*-deficient mutant BEttsI grown in the absence (Ge-) or presence (Ge+) of the inducer flavonoid genistein (10 μ M) after 4 and 24 h. The expression level of each gene was normalized relative to the *atpD* gene (ATP synthase) using the $\Delta\Delta C_t$ method. The values are means of triplicate measurements, and the error bars indicate standard deviations. Statistical analysis by Fisher's method was performed to compare the relative expression levels of *innB* in USDA61 and BEttsI in the absence/presence of genistein after either 4 or 24 h. Means followed by different letters at the same time point are significantly different at the 5% level.



Supplementary Figure S3. Immunodetection of NopA and InnB in extracellular proteins from rhizobial cultures in presence of genistein. Proteins were separated by 4-20% and 4-15% SDS-PAGE for (A) and (B), respectively. Size-marker molecular masses (kDa) are shown on the left. Arrowheads indicate protein bands that were visible in cultures of USDA61 and BE53 but not BErhcJ.



Supplementary Figure S4. Symbiotic properties of *V. radiata* cv. KPS1 inoculated with the rhizobial strains USDA61, BE53 and the *innB*-complemented mutants derived from BE53, designated BE53::innB-1, -2, and -3. (A) Nodule number, (B) nodule weight and (C) plant weight were measured at 40 dpi. At least 3 different *innB*-complemented mutants of BE53 were inoculated with KPS1 in order to confirm the symbiotic phenotypes. Open bars and closed bars in (A) show the numbers of small nodules (<2 mm) and large nodules (≥ 2 mm), respectively. Data showed are the mean of 6-8 plants and the error bars indicate standard deviations. Means followed by different letters are significantly different at the 5% level.



Supplementary Figure S5. (A–C) Infection threads and root hair curling of *Vigna radiata* cv. KPS1 inoculated with *Bradyrhizobium elkanii* USDA61G and BE53G at 8 dpi. Scale bars: (A) and (C), 200 μ m, (B), 50 μ m. (D–G) Thin sections of KPS1 nodules infected by DsRed-tagged strains of USDA61 (USDA61Ds) and BE53 (BE53Ds). (H) Numbers of reproductive rhizobia recovered from nodules. The KPS1 nodules harvested at 30 dpi were used to photograph and count the number of rhizobial cells after surface sterilization. Data shown are the mean of seven nodules selected from seven different plants, and the error bars indicate standard deviations. Means followed by different letters are significantly different at the 5% level. Scale bars: 500 μ m.

2.2 Supplementary Tables

Supplementary Table S1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics ^a	Reference
Bacterial strains		
<i>B. elkanii</i>		
USDA61	Wild-type strain, Pol ^r	USDA ^b
BErhcJ	USDA61 derivative harboring insertion in <i>rhcJ</i> encoding a membrane protein of the type III secretion apparatus, defective in type III protein secretion, Pol ^r , Km ^r , Tc ^r	(Okazaki et al., 2009)
BEttsI	USDA61 derivative with a deletion/insertion in <i>ttsI</i> , a positive regulator of the <i>tts</i> system, Pol ^r , Sm ^r , Tc ^r	(Okazaki et al., 2013)
BE53	USDA61 derivative carrying a Tn5 insertion in the <i>innB</i> gene, Pol ^r , Km ^r	(Nguyen et al., 2017)
USDA61Ds	USDA61 derivative containing a pBjGroEL4::DsRed2 plasmid insertion, Pol ^r , Km ^r , Sm ^r /Sp ^r	This study
USDA61G	USDA61 derivative containing a pCAM120 plasmid insertion, Pol ^r , Km ^r , Sm ^r /Sp ^r	This study
BE53Ds	BE53 derivative containing a pBjGroEL4::DsRed2 plasmid insertion, Pol ^r , Km ^r , Sm ^r /Sp ^r	This study
BE53G	BE53 derivative containing a pCAM120 plasmid insertion, Pol ^r , Km ^r , Sm ^r /Sp ^r	This study
BE53::innB 1, 2, 3	BE53 derivatives complemented with plasmid pBjGroEL4::proinnB, Sm ^r , Pol ^r , Km ^r	This study
BEB759F	USDA61 derivative containing the <i>innB</i> -3xFLAG fusion integrated in the chromosome, Pol ^r , Km ^r	This study
BEttsIB759F	BEttsI derivative containing the <i>innB</i> -3xFLAG fusion integrated in the chromosome, Sm ^r , Pol ^r , Km ^r	This study
BEB759C	USDA61 derivative containing the <i>innB</i> - <i>cya</i> fusion integrated in the chromosome, Pol ^r , Sm ^r , Tc ^r	This study
BErhcJB759C	BErhcJ derivative containing the <i>innB</i> - <i>cya</i> fusion integrated in the chromosome, Pol ^r , Sm ^r , Tc ^r , Km ^r	This study
<i>E. coli</i>		
HB101	<i>recA</i> , <i>hsdR</i> , <i>hsdM</i> , <i>pro</i> , Sm ^r	Invitrogen
S17-1	<i>thi pro hsdR⁻ hsdM⁺ recA</i> RP4::2-Tc::Mu-Km::Tn7(Tp ^r /Sm ^r)	(Simon et al., 1983)
Plasmids		
pRK2013	Helper plasmid, ColE1 replicon carrying RK2 transfer genes; Km ^r , tra	(Figurski and Helinski, 1979)
pCAM120	mTn5SSgusA20 (<i>Paph-gusA-trpA</i> ter translational fusion) in pUT/mini-Tn5; Sm ^r /Sp ^r , Ap ^r	(Wilson et al., 1995)
pBjGroEL4::DsRed2	<i>dsRed</i> transposon delivery vector, Sm ^r /Sp ^r	(Hayashi et al., 2014)
pBjGroEL4::proinnB	pBjGroEL4 carrying a ~2.6-kb DNA fragment containing <i>innB</i> , its upstream <i>tts</i> box and promoter, Ap ^r	This study
pK18mob	Cloning vector, pMB1 oriV, <i>oriT</i> , Km ^r	(Schäfer et al., 1994)
pK18mob3xFLAG	pK18mob containing the 3x FLAG, used for the generation of translational fusions with the 3x FLAG, Km ^r	This study
pInnB7593xFLAG	pK18mob3xFLAG containing a ~0.5-kb DNA fragment from the stop codon of <i>innB</i> thereby generating an <i>innB</i> -3xFLAG fusion at the 3'-end, Km ^r	This study
pSLC5	pSUPPOL2SCA containing the <i>cya</i> reporter gene, used for the generation of translational fusions with <i>cya</i> , Tc ^r	(Wenzel et al., 2010)
pSLC5Sm	pSLC5 containing 1,536 bp of Sm resistance gene (<i>aadA</i>) and its promoter in <i>DraI</i> site, Sm ^r , Tc ^r	This study
pInnB759Cya	pSLC5Sm containing a ~0.5-kb DNA fragment from the stop codon of <i>innB</i> coding region thereby generating an <i>innB</i> - <i>cya</i> fusion at the 3'-end, Sm ^r , Tc ^r	This study

^aPol^r, polymyxin resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Tc^r, tetracycline resistant; Tp^r, trimethoprim resistant; Ap^r, ampicillin resistant.

^bUnited States Department of Agriculture, Beltsville, MD.

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Supplementary Table S2. DNA oligonucleotide primers used in this study.

Oligonucleotides	Sequences	Source	Usage
3FLAPstI	5'-GGATTACAAGGATGACGACGATAAGGACTATAAGGACGATG ATGACAAGGACTACAAAGATGATGACGATAAATAGGCATG-3'	This study	Construction of pK18mob3xFLAG
3FLASphI	5'-CCTATTTATCGTCATCATCTTTGTAGTCCTTGTCATCATCGTC CTTATAGTCCTTATCGTCGTCATCCTTGTAATCCTGCA-3'	This study	Construction of pK18mob3xFLAG
aadAfor	5'-TGATTGCTGTTACGGTGA-3'	This study	Amplification of Sm resistance gene
aadArev	5'-TACTGCGCTGTACCAAATGC-3'	This study	
BeatpD-F	5'-GGTCGTCGATCTTCTTGCTC-3'	This study	RT-PCR assays
BeatpD-R	5'-CGGCGAACACGGAGTAAC-3'	This study	
BenodC-F	5'-CGTGGAAAGCCAAGTGATTT-3'	This study	RT-PCR assays
BenodC-R	5'-TTGCTGTCGCAGATATGGTC-3'	This study	
BenopA_F	5'-CGCAGCTGGTACTGCTACTG-3'	This study	RT-PCR assays
BenopA_R	5'-GAGACGACGCGAAGTTCTACA-3'	This study	
BeinnBRT-F	5'-TTTGC GCGATGATCTATTCA-3'	This study	RT-PCR assays
BeinnBRT-R	5'-GGACTTGCTACTCCCTCCAA-3'	This study	
BeinnBSacI-InfuF	5'-GGGAACAAAAGCTGGAGCTCAGGAACGCTTACGGGCTGTCTTCTC-3'	This study	Complementation of <i>innB</i>
BeinnBKpnI-InfuR	5'-GCTAGGGCGAATTGGGTACCTCAGCCACGTTACAGGATAGATGCGA-3'	This study	
PsuppolF	5'-ATAAACCCAGCCAGCCGGA-3'	This study	Confirmation of integration of <i>cya</i> fusions
PsuppolR	5'-TTCTGACAACGATCGGAGGA-3'	This study	
M13 forward (-20)	5'-GTAAAACGACGGCCAGT-3'	This study	Confirmation of integration of 3xFLAG fusions
M13Rev0	5'-TTCCGGCTCGTATGTTGTGT-3'	This study	
Be53-F	5'-ACCTTGCCATCACGATTAGC-3'	This study	Confirmation of integration of <i>cya</i> and 3xFLAG fusions
Be53-R	5'-GCCTCGGTCATCAAACATCT-3'	This study	
innB-PstI-fullF	5'-CCTTGTAATCCTGCAGGCCACGTTACAGGATAGATGC-3'	This study	Construction of the <i>innB</i> - 3xFLAG fusion
innB-BamHI-fullR	5'-CGGTACCCGGGGATCCCAGGAAGTGC GGGATATTG-3'	This study	
aadA-DraI-F	5'-ACTTTAGATTGATTTAAATCCAAACGAGAGTCTAATA-3'	This study	Cloning Sm resistance gene
aadA-DraI-R	5'-CACCTAGATCCTTTTAAATTGACCTGATAGTTGGCTGTGAG-3'	This study	
innB-EcoRI-fullF	5'-GATGCCCCGGGAATTCCAGGAAGTGC GGGATATTG-3'	This study	Construction of the <i>innB-cya</i> fusion
innB-XbaI-fullR	5'-TTGCTGCATATCTAGACAGCCACGTTACAGGATAGATGC-3'	This study	