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**Supplemental Information**

**Specificity and Promiscuity at the Branch Point**

**in Gentamicin Biosynthesis**

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## SUPPLEMENTAL INFORMATION

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**Figure S1, related to Figure 2. Sequence alignment of GenK (A) with homologous radical SAM-dependent methyltransferases and of GenQ (B) with homologous oxidoreductases from other aminoglycoside biosynthetic pathways**

Black letters in gray boxes represent amino acid residues identical in all species, while white letters in gray boxes represent similar amino acid residues. The red box in **A** indicates the consensus CX<sub>3</sub>CX<sub>2</sub>C binding motif for the FeS cluster.

**A**

GenK	1	-----MNALVAAPSVTEGNQVKVFLVKPPIRGCMVEIGRHVPIGLAYVSSALRAAGHETEIFDS-----LAYTEDNHVVPDAEL
ForK	1	-----MSTRTD TG VTRVLLIKPPIRACMIEIGRHMP IGLAYLAAQLRNSGMEVDIFDS-----LAF SEDNHVVP SQY
Fom3	1	-----MTIGSLGSTE FALHGKPAIRWGDLPQRVGGKASRRYQKVL LNP SATLFRHDLPRCTYPLGLGYIAAVLEKYDYEVVKIL
GenD1	1	MTVTNKIVTGVAFPPSLLAETPPISVATLTAYLRDKGMPAVGLDLNADFNEYLLNRVEIEQVQGPENTHEFTKPF IKQDFLNIITGNYTE
GenK	84	RHPR--WRHIDHIG-----ARTERTEAATAAS-----EADVVGISCFPTPYYESAYELARAKRVLN
ForK	78	AHPR--WDHLVHIG-----ADWARVEQVRR-----GYD VVGVSCFPTPYEPAYELGR AKQILPQ
Fom3	89	AQPVDGDEQFLRIG-----LSDDDIVKVIKEF-----APDVI GISSFNSQADNVHLLK ADLVAPE
GenD1	101	QQCQVAPESLSLIDPPFPFSYCEFLSILRDEPERVAKLRDPPDANIYHAFYQEKVAGKASELGLHGFSLIGYNQVIPALTLGYMKKENED
GenK	149	GTVAFFHVIE-----VPEVDAMICEAEVTVALLDAFATGRPLTEILG-----VAFRCGEG-----LCECATPGTPHIR----
ForK	142	PTVAHPHATA-----EEAFDAVVICAEANVVEIVLAAGRSLRGLPG-----LTFRCGTG-----LCD CPRPSGVHLQ----
Fom3	156	ARYFPKACID-----DPNLDAVVICGEMTFLWLPHLNGNVREDEVHG-----LAWRDRDGKI QIKPELPLISSMRPEGPEQGK----
GenD1	201	VTSEADMLIPRLEACEPELGELIDAVVREGEPELAKMAEALSRGERPVGIPGGCKPMSEQSVLDTKPVGRKLLAMSQPRENMPNTHWRIE
GenK	216	APFVADLDSAPFAADQLDFDRYGNAVT-----LITSRGCP-FSCSFCVHATVG-KQFRARDPQRVDEDEHYVNVHGVRRF
ForK	209	AEFLQDLDCALBAVDLLMGSYDETAT-----LITSRGCP-FSCSFCVHATVG-KKFRARAPENVDEDEHYVTEHGIRRF
Fom3	235	LSMAGELDHPPFAWHYMEKYFEIKAYQSPYTVGSRVQGLYTSRGCT-ANCTFCITTFHWG-QKLRRSVDNVVNEFLRLRDEYGDDEF
GenD1	301	ISWVADMHQPTFDYSDFL SLYTTFRE-----GQGSVLVQGSRSQYMKCSFCNAITNFAPWSYREKSTENIQKIDTFLELM-PGTV
GenK	301	DIERVHATCQEI VRRKLDVRLSLPNGMTVVKITDLESVVSIGFDDIFLGETTDAARLRKRRKGFTS DKVSAGVALFEKGLTASAAI
ForK	294	DIARVHEICRAITRRGLDVLEHL PNGMTVVKIDPLVDDMAAGFQSIFLGETTDVKRLRQRRKGFTS DEKVNAGAGLFDHGITVGSAL
Fom3	333	DMDHARELFRAFK--EVGLPWATPQGTALWRDDEL DLMAESGAYQVFAAESGVQRVLKDIKKPLNIGRTSHLIKYARSLGMHVHGFF
GenD1	393	PAKRLVELADFFIAKKRPELFWVDVRFEGNIDAVLTKMRDS-QGTRFGLETANERLLDLRRKGNR-DEVVHRLLD SRELGYKPFIMT
GenK	399	SLDAIVQDAVNLVLGVEW TNPFPYIYGSPDYQTC LTRGIVDPLTDPALFDQ-FNFAFANGVLAAD ELYTAWVGTLAWALWPKYVLEGA
ForK	392	TPAEVARDSNLMLAGIRW TNPFPYIPGSPDFQQCLANGLITHDTLALYDQ-FNFAIGSDHLSPEALYWSVVITQAWAHWPDYVLEGA
Fom3	427	PMFNGAGESHEEMQASYDAE-----ESGFSSASFFFAASPIVGSSEL-LRECI RQGFVDP-EESLYRMTYKQCHINVPGLWDGE
GenD1	491	EEAEELYQFLSDYHDVTYQIADFIVERNSEPIQLRPDDYGIHIDDEQESFHNNLHFTRRAGYSDEEAQEVYRDLVVRTQRFKGAHEVDV
GenK	496	SAAVAGARLVEHSMQLDPESPEELPATVRAIRESADGLLALGHPLGCVCMQHVAADADKAGADQFCRFAGDMIAAAIALYSGQPQVSAQ
ForK	490	GLDVALQRLLAHSDSLFGNGELEVPAVP--GRSTATSCTATRRLPRGAAHQPAATARRGLHLHR
Fom3	503	-IADLAAKFNRFNRRDRD-----YTPQKQWNAQY-----
GenD1	589	APDLSVYRLSLRAGSFALENYVVKHNNLPFEGLVPIGYKVVQQTWDMTDKGTVFEIDPDIALGALAGAGSR-----
GenK	596	EGCSFMVRPTGDERIGRIQRRFVELLDENRREAELVTAEAVSH
ForK	553	-----
Fom3	534	-----
GenD1	659	-----

ForK (accession no. CAF31547.1): from *Micromonospora olivasterospora*;

Fom3 (accession no. ACG70826.1): from *Streptomyces fradiae*;

GenD1 (accession no. CAF31434.1): from *Micromonospora echinospora*.

**B**

GenQ	1	-----MTGSGASCAITAWALQRAGLDVVVVEQGPFVDPWVSYDDETVAETAWIR
ForQ	1	-----MTTSDARYGPEPTD-----GDVRYEYDVCVVGGGARCAITAWALLSRAGLSVAVVVEQGGHVTADTSDYDILLAAESAWVR
Neo11	1	MKRLRGITLPSDARHAWHPEPLGPAHRDGDWTRDDDRVWVWVVTGSGASCSVAADRIVRQGLDVLVMEEGFRISPDLGNEPLDDMCRTALAR
LivQ	1	MERLRGFSPLENTTARHPAPLGAHRDGLPEGTADRVWVVCVTGSGASCSVAADRIVRQGLDVLVMEEGFRISPDLGNEPLDDMCRTALAR
BtrQ	1	-----MSHAEKAYAR
KanI	1	-----MRIISVQEAARTFDVVCVVGGSGASCAITAAVLAERGLSVLILEQGTATPPGTDHDDVEDPDTHAYAR
TobQ	1	-----MLLISADEAARRTYDACVVVGGSGASCSVAAAVLAERGLSVLIVVEQGGPFPAGADVEDHEDPDGWAFAAR
GenQ	60	GNPWSTCNVGGGTVFYGGAAFRYRPVDFDAESRGRSDLPLRNPWVDFLDFYYEYFELALGTSG--GGHDPISLPS--NPSYMPRPVETTAEG
ForQ	85	GSPWTTCNVGGGTVFYGGVLPFRHRPIDFDPERVGGQADLPLRNPWDFDLEFYTAVEDIIGVAGLAGADPGIPARSAPIPARAGAHLAG
Neo11	101	GWPWTTSNLGGGTVFYGGASRYRPFDFDPSELVDAGGLDVRWPYGLAFLAPYYDVLERRLGVCG--GEE-----GEGSRGPAHPPTAA
LivQ	101	GWPWTTSNLGGGTVFYGGASRYRPFDFDPSELVDAGGLDVRWPYGLAFLAPYYDVLERRLGVCG--GDAPGIHR--GSRHSRGAHPQPSA
BtrQ	20	GYPWASAVGGGTFYAGISRYRQVDFDAREYASDALDPWPIGYSLLDFYYNKTEQWLGVSGDLNADPFKNS--AGTGLRTHAHSYSQQ
KanI	76	GYPWASAVGGGTVFYGGISRYEQDLDPPPALGDADYAHWRLRLDLEEHYDWVEDRLGVSG--PSHGRVGYAFPHYARGSLPHTPM
TobQ	76	GFPWTAMAAGGGTVFYAGISRYRQDLDLAPPSSLLGEADYDHWQVGLALEPHYEWTEYQLAVAG--PSRKRKVDYVFPVYHRPSLPTTFP
GenQ	157	SLGLNPFPTPLAATESYHGRLACAGERPCISNRCERGAAGDAITVFLDFE--ARKAGLRFAGLKAVRLLRRDATSVDDTECVRVNDGNRHV
ForQ	185	ALCWHFPTPLGLTDAYRHHGCVADAPCISRTCPVGAAGDAVNRFLTPE--ALRAGARFAGLKAVALLGDERHDARARCVRVDTGRWYE
Neo11	192	ALGYRFPPTPLANRHAHGGRSACERNLSVSHQCSGTAKGDAVAVFLAPLAHPNFTRTGVTAARLNQDRPDAVGSVTCODRLGRITHR
LivQ	198	SLCYRFPPTPLANRDPHGGRAAACARDSLCVSHLCPGTAKGDAVAVFLAPLAHPNFTRTGVTAARLEQDRSGEVAAVRCODRQGTQAHR
BtrQ	119	ELGLHFPTPLAINTDHNYPGCDNLSTCTDYACPIGAKADSFTRILKPVKLEHVTMKNTAFAFHQTDPSVDSLECDLDTKERH
KanI	174	ALGLTPLSTPLASGARDRHGPGCAELTPCTGFTCPVNAKADVSRILAR--AEGDVSALDTRARFVASAPGRAKRELVGGSPRSRS
TobQ	174	ALNLTPLTPEVALSGIRDRFGPGCAQATPCTSFSCPTGAADVSRVLAPE--AEGDVSALVHTVDRLVASGSHRVDAAEVDRGTGTRRT
GenQ	256	GNAVQSAALLLRSTDEASFGGNEHDLVGRGLCFKMSGYVLGYRRDTPVTPGGGRVAGPSPS--ATITDVTYADDAFGGEGVLEFSRP
ForQ	284	GNAVQSAALLLRSTTERHERGLGNSHDLVGRGLCFKLSYVLYGHRGSDEPPRS--EVMGLGPVSAATITDFYEDPAAGGGEGVLEFVVR
Neo11	292	CNAVQSAALLLRSGRGAEDGFGNHSGLVGRGLTKMLSEYVSVVDAPSAATLAD--WRAHAGPSTIAFLDLYLDADCEGEGGTYE SKN
LivQ	298	CNAVQSAALLLRSRTPYSEDGFGNHSHLVGRGLCKLSEYLSGTVDA--DPAVLAD--PYTNTGPPSVAFLDLYLDPDCGEGGGLIYESKR
BtrQ	219	GNAVQSAALVLRSTNKWEDGFGNKSGLVGSGLSFKNSEYVSVVDVMDHPEYQLNE---PLKGLYSVSTIDYQDPHCHSGEGGTYE ANP
KanI	272	ANAVQSAALLLRSDRREDFGFGNSGQVGRHLAMKNSVYVRRGTQERIVAHQPL----RHRYSVVCVLDLIRGAEFEGGGLIYES ANP
TobQ	272	ANAVQSAALLLRSTRLEDFGFGNGLVGRHLAMKNSVYVRRGVPVRRPLGYTPL----RHRYSVVCVLDLIRGEFEDGEGGGLIYES ANP
GenQ	356	EQ--IIRLECIYVDPQRAENRVTLGRVDQFQPDVVDYSPHERDRARLEYLQQAEGLRAAGCELTWRESYWMGSHLHGTCRAGT
ForQ	383	EQ--LLRIEALVDFEPQPTNRVRLGTGTDCHGVTDVVDYQAHPRDRARLEYLRRGEEELRAGCSVVVREPSSGELGSHLHGTCRMGT
Neo11	391	VL--ELRIETIADHEFLDNRVRLSSHADEGEPAAVVDYTPDPRDLRRLAYMTDVCERLRKAGATGIAHEESGAAQSSCHLHGTCRAGD
LivQ	396	AL--ELRIETIADHENLDRVGLSTHLEDEGEPAAVVDYTPDPRDLRRLAYMTGRCERLRKAGARGIRSRSTGAAQSSCHLHGTCRAGH
BtrQ	316	GM--YVQLECIHADQPMVTNRVRLSNDKATGVPKVDYQTHEWKKRLESMTEPAGELTAMGAKRVQCHPYSYLLGSAHLHGTCRAGT
KanI	367	GAGSLIQLECLIGDRFQARNRVRARSRRDGGQRVVDYRQHPLDGERLDVLQGAQDVLHAGARTESVDSATGSHLHGTLRAGD
TobQ	367	ERGTVLQVECLIGDRFQARNRVRLSRTRDRDFFRVLDYEPHADLRLAVLSEATDILTRGAIDVREVDVAELGSAHLHGTLSER
GenQ	454	DGRVHGLTNMVVDGGVMEYFGGVNPTLTIQALALRMAQDLRREFG-----IDPDFSL-----
ForQ	481	NGRTHDADNYVADGALLEPQAVNP TLTIQAVLRVAQRVLVDRFGAPATPVGEVVSPPTVVVVPAQRSAPPATLPHG
Neo11	489	WGRVHSAPNYVVDGGFMEYFGGLNPTLTIQAHALRSKAVAGDLVS-----RHTAHV-----
LivQ	494	WGRVHSADNYVVDGSMFMEYFGGLNPTLTIQAHALRSRAVASHLAA-----DRAAHV-----
BtrQ	414	FGRFHTIDNLEAADGSFMEYFGGVNPTLTIQANALRIENMSV-----
KanI	467	TGRTHDYDNYVADGATFPEAGNFP TLTIQANARRIAVGTS-----
TobQ	467	FGRVHGYDNYVAVDGAATFPEAGNPNPTLTIQANARRIASAD-----

Neo11 (accession no. CAH58694.1): from *S. fradiae*;

BtrQ (accession no. BAE07079.1): from *Bacillus circulans*;

LivQ (accession no. CAG38701.1): from *S. lividus*;

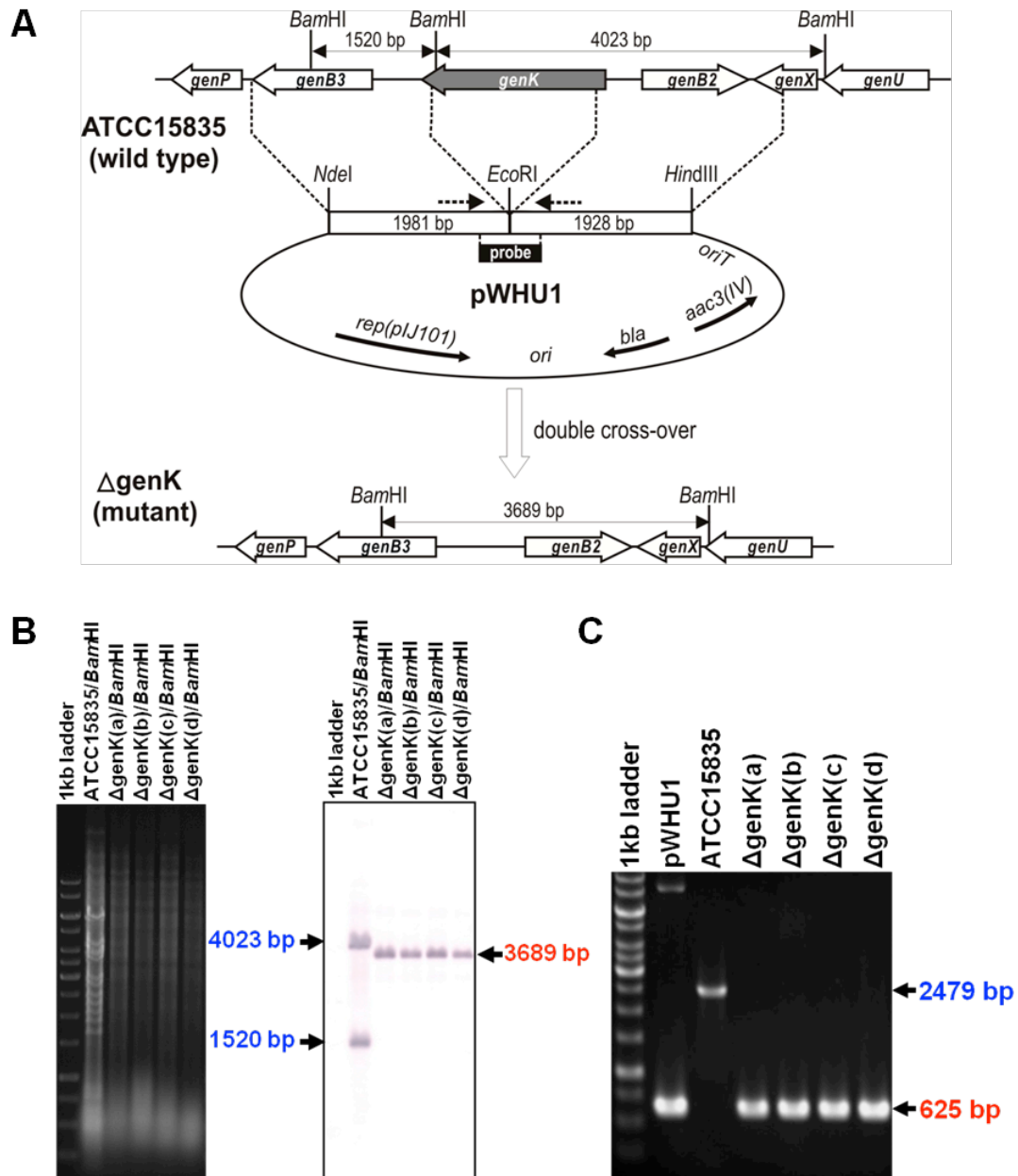
KanI (accession no. CAF60534.1): from *S. kanamyceticus*;

TobQ (accession no. CAH18553.1): from *Streptoalloteichus tenebrarius*;

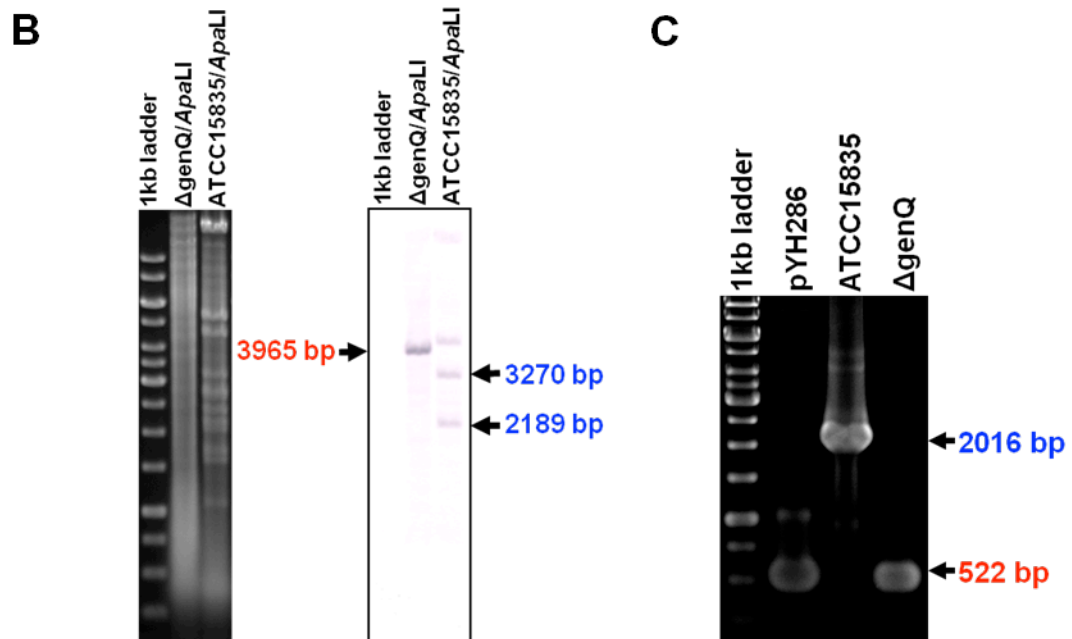
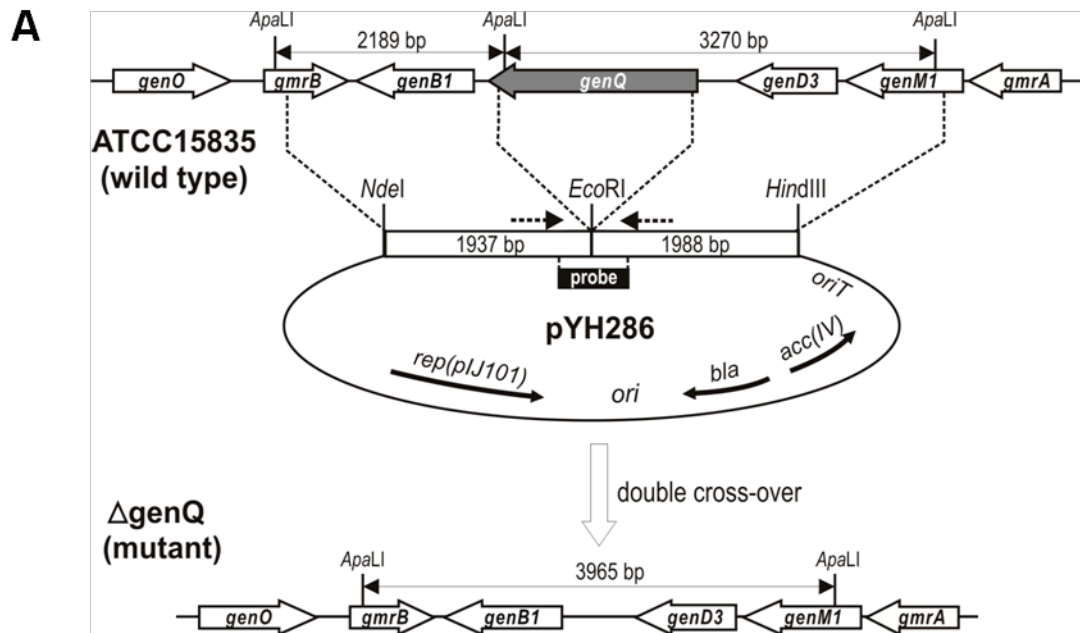
ForQ (accession no. CAF31536.1): from *M. olivasterospora*.

**Figure S2, related to Figure 2. In-frame deletion of *genK* and *genQ* in *M. echinospora* ATCC15835, and complementation of  $\Delta$ *genK* and  $\Delta$ *genQ***  
 (A) Schematic representation of the in-frame deletions; (B) Confirmation by Southern blot; (C) Confirmation by PCR. The arrows indicate the expected size of the PCR fragments in the wild type and mutants. The dash arrows show the location of PCR primers for check mutants.

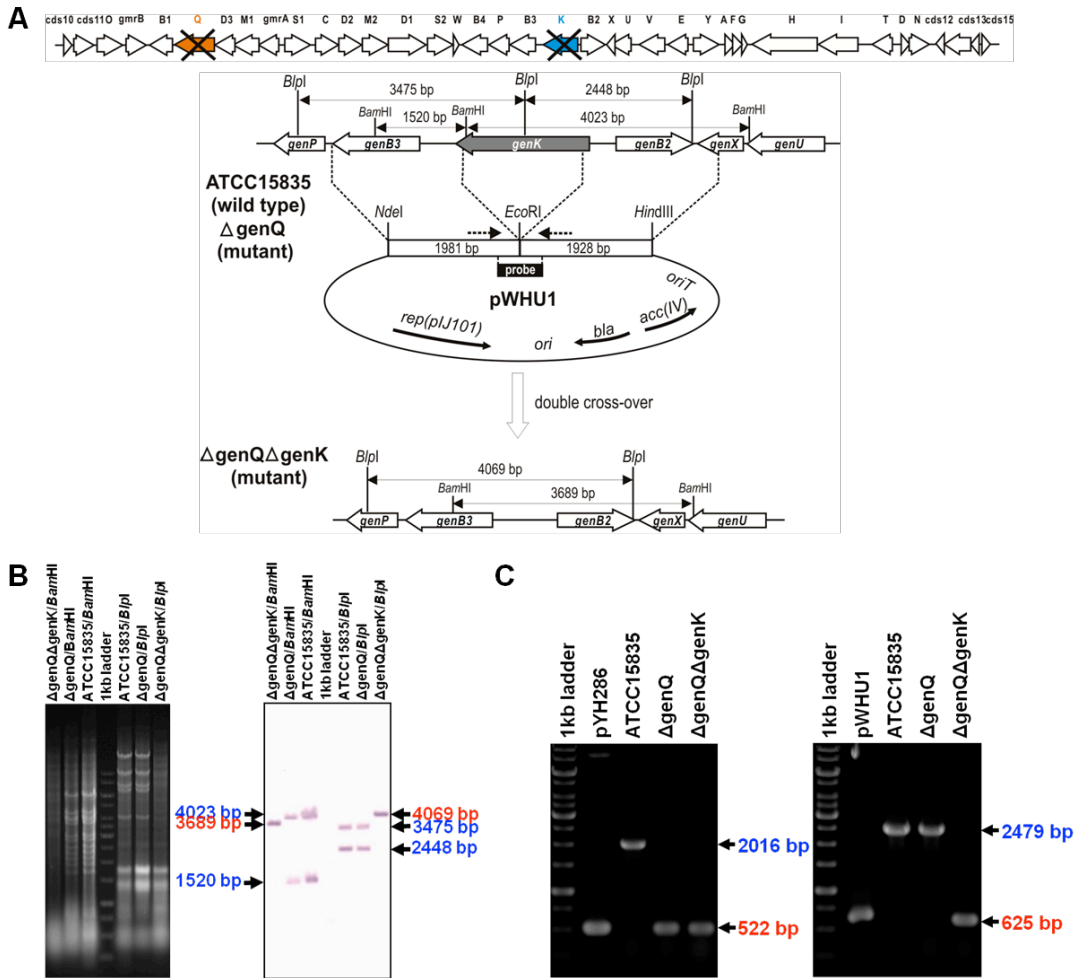
**(1)  $\Delta$ *genK***



## (2) $\Delta$ genQ

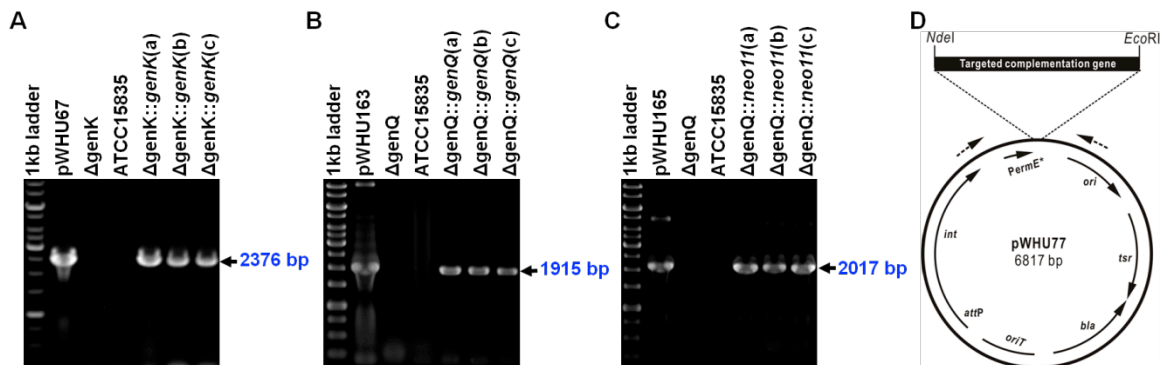


### (3) $\Delta$ genQ $\Delta$ genK



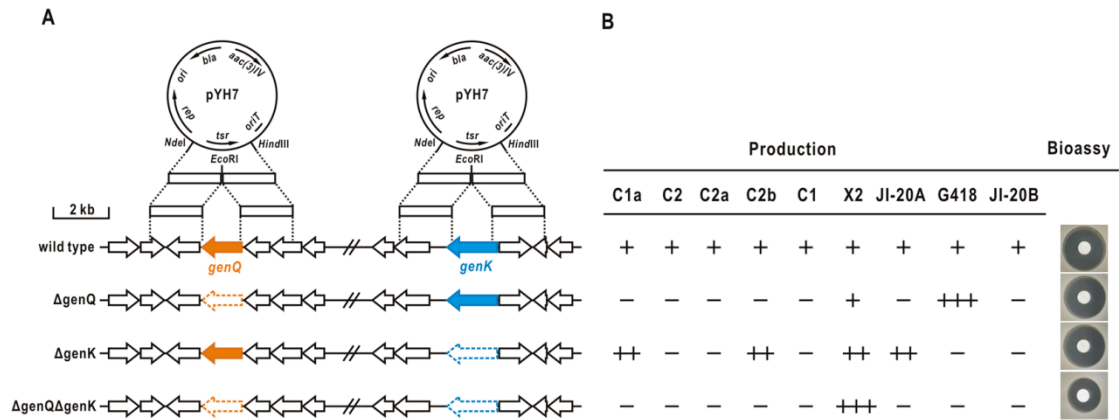
### (4) Complementation of $\Delta$ genK and $\Delta$ genQ

Confirmation of complementation strains (A)  $\Delta$ genK::*genK*, (B)  $\Delta$ genQ::*genQ* and (C)  $\Delta$ genQ::*neo11* by PCR. The arrows indicate the expected size of the PCR fragments in the wild type and mutants. (D) Map of genetic complementation vector. The dash arrows indicate the location of PCR primers for check complementation strains.



**Figure S3, related to Figure 2. LC-HRMS analysis of production of gentamicin C complex and intermediates by the wild type,  $\Delta$ genK,  $\Delta$ genQ, and  $\Delta$ genQ $\Delta$ genK mutants of *M. echinospora* ATCC15835**

(A) Schematic representation of the in-frame deletion of *genQ* and *genK*. Deleted genes are shown in dotted arrows. (B) Production of gentamicin C complex and intermediates analyzed by LC-ESI-HRMS and bioassay using *Bacillus pumilus* as indicator strain. -: no production, +: production, ++: increased by about 10-fold compared with wild type, +++: increased by about 100-fold compared with wild type.





**Figure S4, related to Figure 3.** (A) Sequence alignment of GenB1, GenB2, GenB3 and GenB4 with their homolog Neo18 from the neomycin biosynthetic pathway. The red box in (A) indicates the putative catalytic lysine; (B) Schematic representation and confirmation by Southern blot of in-frame deletion of *genB1*, *genB2*, *genB3* and *genB4*. (1) ~ (4): Single mutants; (5) ~ (10): Double mutants; (11) ~ (14): Triple mutants; (15): Quadruple mutant.

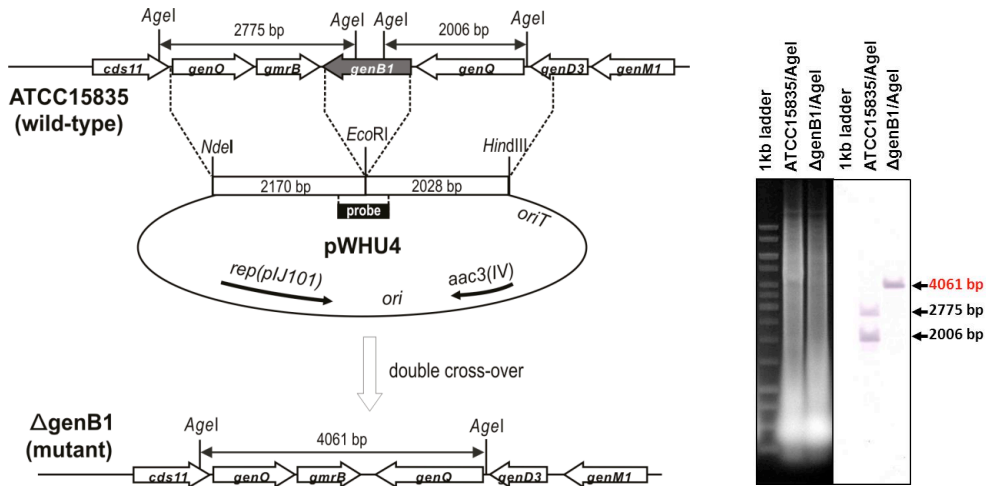
**A**

Neo18	-----MTKNS---SLLAEF	11
GenB1	-----MTIDIGAGKLLAQE	14
GenB2	-----MIIANA	6
GenB3	MAVADHRSSSEPSWRAGRTRARRRSRWHSWARVKSAREGSGQDMSANLTNRGLVERARRVTA	60
GenB4	-----MNYRELIERARRTTA	15
Neo18	PTCFRDEKDRPRVFTAASGAWLTDESDFRWDIFDNARGSILLGHGDPVVAEAVARAAAT--	69
GenB1	PTCFRDADGRPRVFVEGSGAYLTDPDFGRRWIDFNDARGSVVLGHGDEEVAEAIARAA--	72
GenB2	DGCTPEYVARGVTIVRGEAYVYDAEGRGLIDLNSFGSVMLGHQDPVVTEAVLKTVR--	64
GenB3	AENYDIGTRFSAMIQSGEGAWLTDEVEGNYVDLTAASGTTILGHRNQAVTEAITRQIRDF	120
GenB4	AEEYDISGRYPSVIAHAEGAWMTDLSGNRYVDLTGADAVALGKYPVAVNEAITRQIRDY	75
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Neo18	GADGTATGWSRRVDAVLERLHALCGGEVVGFLRSGTAAVRAAVLAVREATGRPLLLSAGY	129
GenB1	GRSGVGTAWSPVLDSLGLQEQVCGGDVGLYRGTAAALRSVTCVARDARDRSIVLSGYS	132
GenB2	SGVFAAASLDLQNHLEAQIAGDLPDQORVAFFKGTAAATRAAASAAARQVTGKRLIASCGY	124
GenB3	GTAFATLSVPRVELAERLCERYECAEKVVFHKGTSEGTAARLARARTGRELILSCGY	180
GenB4	GTTFATLSVPRVELAERMCCERYECAEKVVFHKGTSEGTAARLARARTGRELILSCGY	135
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Neo18	HGYDPMWYSEAPLEPNADGVDDFFDLGLLRELLRA-PERVAVVSPDHMHLSPGWYR	188
GenB1	HGYDPMWH-CDEPFTPNQHGIVEFLFDLDVLAEWLSR-PEQVAVVSPDHMHLSGERWYT	190
GenB2	HGYDLMWE-FTPPGQPNSEDLVLCYHLP ELIDQVLDKHAHELAAVIIAPDYIHVSP EYIA	183
GenB3	HGWHEWQLAGETFGYQQTGTVVGGYNEKALAKMLEAFGNVAVGLISPELLYDFVDFYQ	240
GenB4	HGWHEWQMGAEFFGYQQTGTVVGGYNEKALAKMLEAFGEQVAGVIVSPEVLVDFLDHYR	195
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Neo18	ELRRLCSAAGVVLVADEVKVGLRYAPGLSTAE-LLAPDVVVAKEMANGHAVSAVGGSR	247
GenB1	ETRLTKEADVPVIADEVKVGLRYRAGLSTP--LLDPAVWVYAKLANGSPVAAVGGDAH	248
GenB2	DLFERCERVGVTIADEVKHGYYLRQGA SVTEASVADMVYAKGISNGWPLSCVAGDER	243
GenB3	RMVALCAR YDVPFMMDEVYTGFRAGP-KGVHGLGVPADVVVSKGLANGHSLAAMVGRD	299
GenB4	RMSALCAR YDVPFMDDEVYTGFRAGP-KGVHGLGVPADVVVLGKGLANGHSLAAMVGRD	254
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Neo18	LLKP--LKEVSFTSFFEP TILAAADAALARVATGEPQRAVREAGDRFLRHARKALDDASL	305
GenB1	LLAA--LEDVSFTSYFEP TAMAATTTLRMATGEPQQAIRAAGDRFIAHTRAAFANAGV	306
GenB2	FLKP--LAEFVSTLTFEAP SFAAASATLDRLAELDVAQLAIDGARFVSEAAKMI STRDL	301
GenB3	IIDAYDVS GIQGT YTREVP PMAAAMAVLDVLDTPGVYEHAEAMGRRLADGMREILLTGEI	359
GenB4	IIDAYDVS GIQGT YTREVP PMAAALAVFEVLDTPGVYEHAEAMGRRLADGMREILLTGEI	314
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Neo18	PVEIAGDGTTFQFVPATEELEEAALYGAANAEGLLFYAGDNQGVSAAFDEAVLGEAERRFA	365
GenB1	PIDLAGNGLFQFVCADDEVADAFHAAA AEGLLFEGDNQTPSAAFTDEVVEDACGRID	366
GenB2	PIEMAGTGAFFQFVCA-EVEVEVLLPHALAEGLILEPSDQQYP SACFRGEVVDALERLD	360
GenB3	PNWVGGPALMFDTVLPND DLGWEIYKTAHDFGVYFEDSGTQLVTAFADEAAVDHALTAFR	419
GenB4	PNWVGGPALMFDVLPND DLGWEIYKTAHDFGVYFEDSGTQLVTAFADEAAVDHALTAFR	374
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Neo18	RVCERLAPY--AGGEPVGDAAARYVAWNVMDGLRQAPRDRETTGLLARLLDD-----	416
GenB1	RVSAAITGR---FTDRELTEESWYASAWGAMDGLADRPRTRETTAIVERLWED-----	417
GenB2	RALTTMAAARPDVGREVTQLDRVNAAFQMDGLPGRPDGWSLDQCV EYVTAQL-----	414
GenB3	KATRQVIADRPDIAPTSGGELTEERKLDFAEEAFGLLRDDEIN ALIDETIEKV VNRDR	479
GenB4	KATRQVADRPDIAPTSGGELTEERKLDFAEEAFGLLRDDEIN ALIDETIEKV VNRDR	434
	: . :	
Neo18	-----	
GenB1	-----	
GenB2	-----	
GenB3	SIKPVLP AQN 490	
GenB4	SIKPVLP AQN 445	

## B

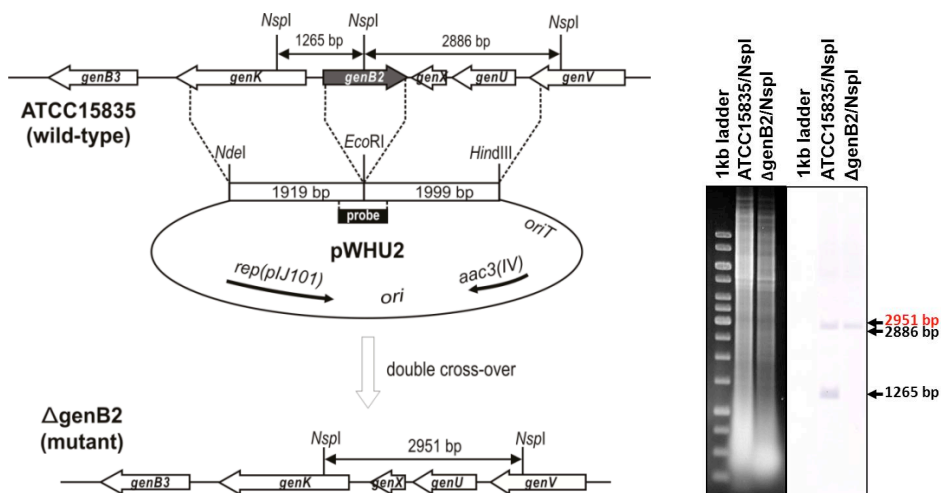
### (1) $\Delta$ genB1

The arrows indicate the expected size of the fragments from the wild type and mutant chromosomal DNA, respectively, hybridized after digestion with *AgeI* with a 581 bp probe (PCR-amplified from pWHU4 using oligonucleotide primers genB1-CK1 and CK2).



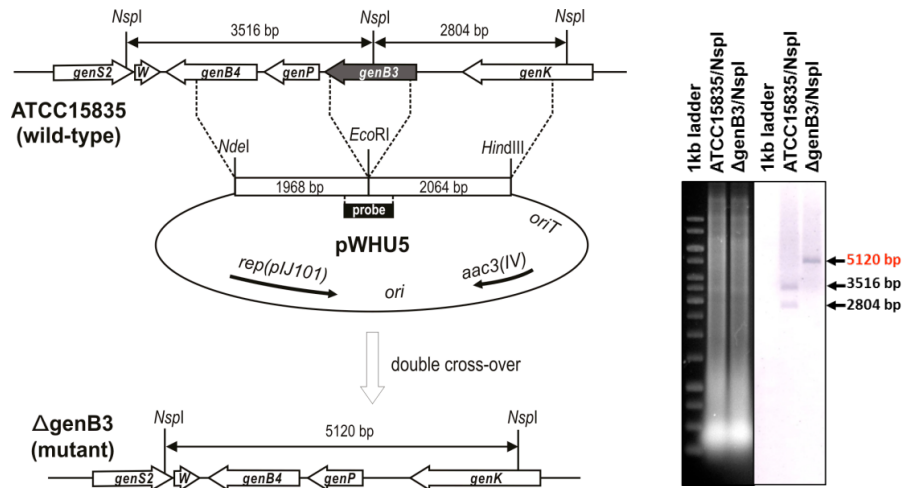
### (2) $\Delta$ genB2

The arrows indicate the expected size of the fragments from the wild type and mutant chromosomal DNA, respectively, hybridized after digestion with *NspI* with a 587 bp probe (PCR-amplified from pWHU2 using oligonucleotide primers genB2-CK1 and CK2).



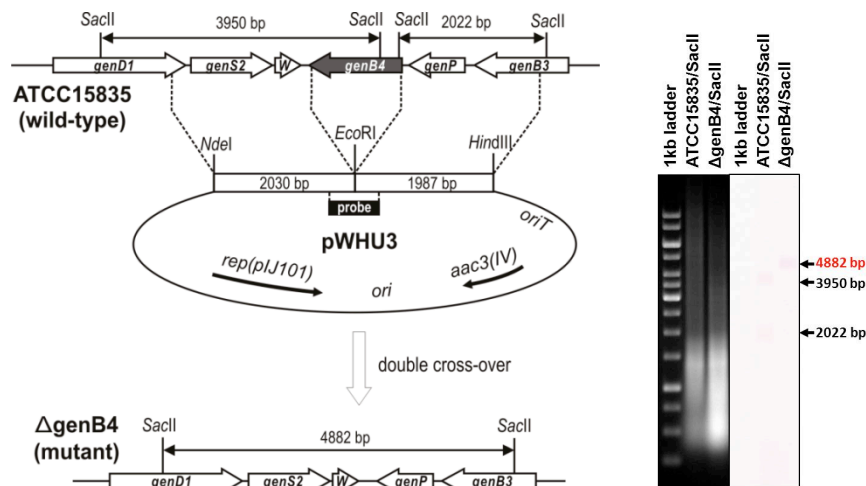
### (3) $\Delta$ genB3

The arrows indicate the expected size of the fragments from the wild type and mutant chromosomal DNA, respectively, hybridized after digestion with *NspI* with a 658 bp probe (PCR-amplified from pWHU5 using oligonucleotide primers genB3-CK1 and CK2).



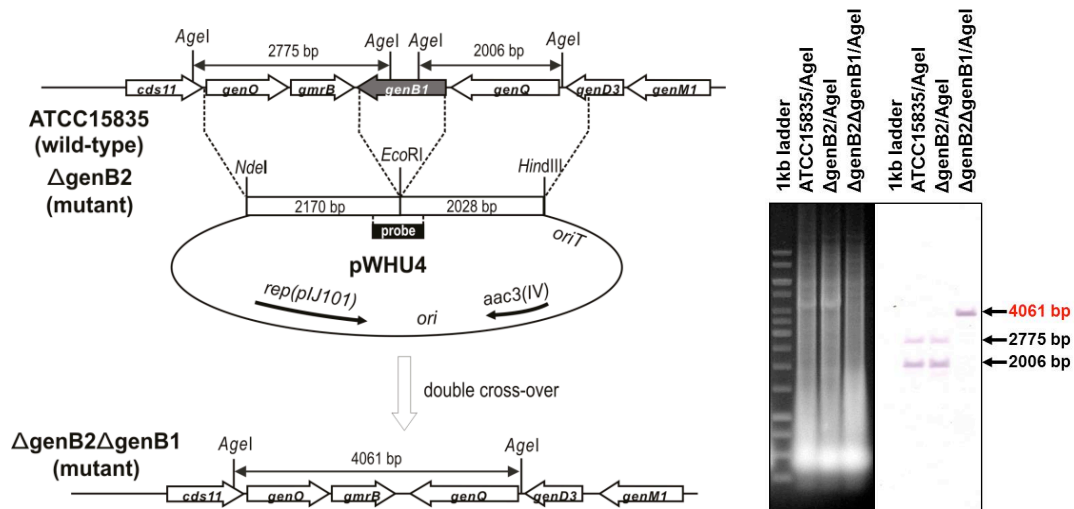
### (4) $\Delta$ genB4

The arrows indicate the expected size of the fragments from the wild type and mutant chromosomal DNA, respectively, hybridized after digestion with *SacI* with a 595 bp probe (PCR-amplified from pWHU3 using oligonucleotide primers genB4-CK1 and CK2).



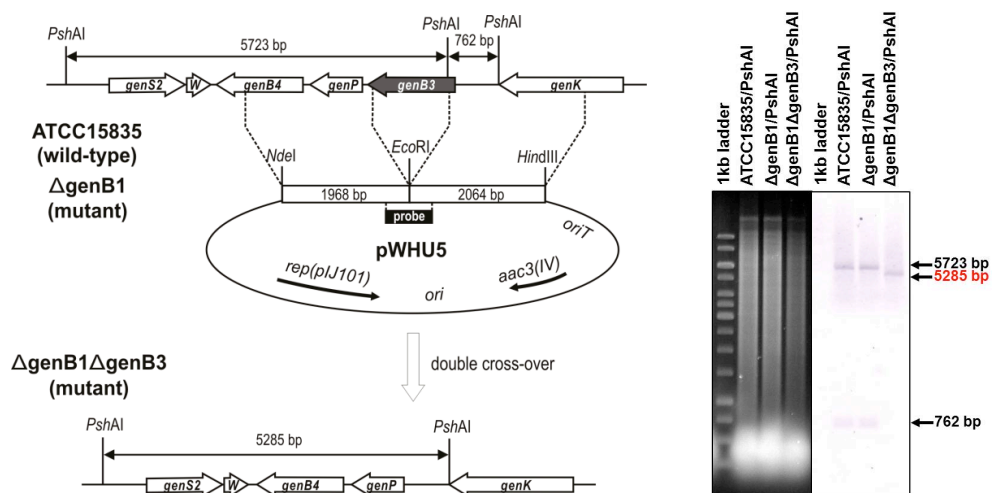
### (5) $\Delta$ genB2 $\Delta$ genB1

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *AgeI* with a 581 bp probe (PCR-amplified from pWHU4 using oligonucleotide primers genB1-CK1 and CK2).



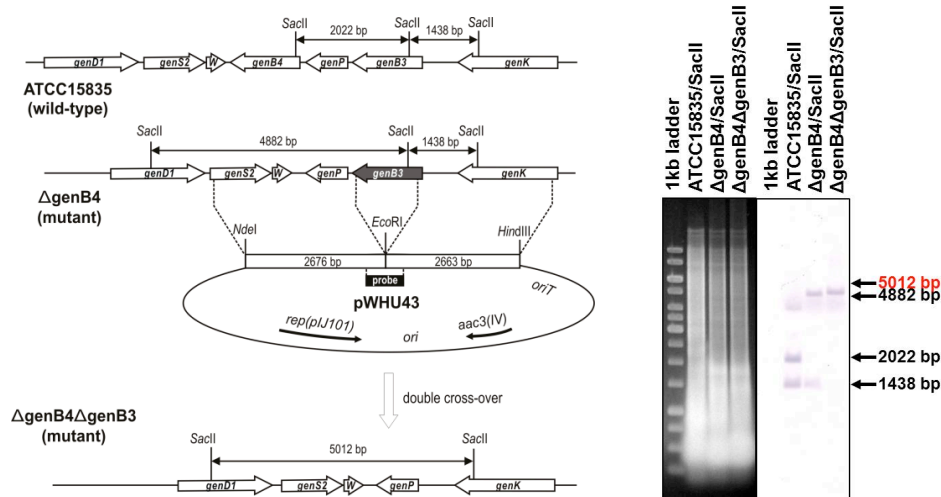
### (6) $\Delta$ genB1 $\Delta$ genB3

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *PshAI* to a 658 bp probe (PCR-amplified from pWHU5 using oligonucleotide primers genB3-CK1 and CK2).



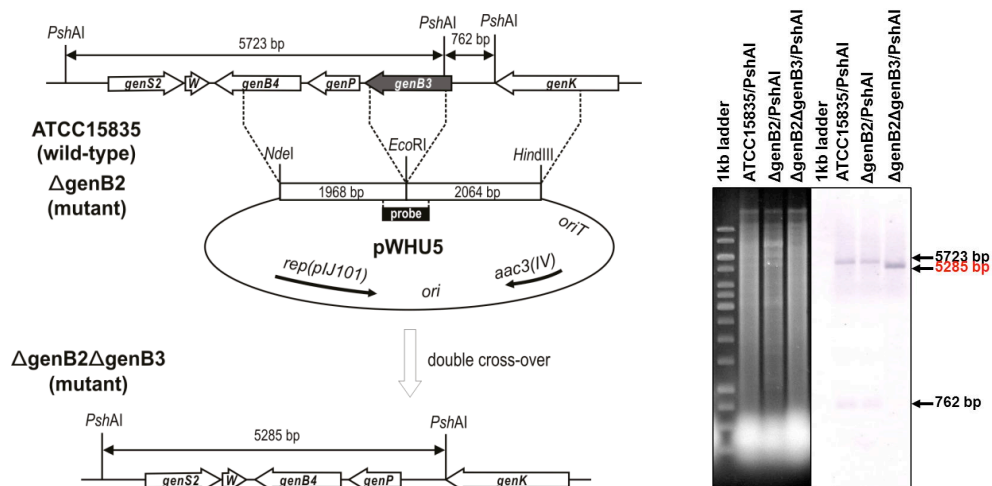
### (7) $\Delta$ genB4 $\Delta$ genB3

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *Sac*II with a 741 bp probe (PCR-amplified from pWHU43 using oligonucleotide primers genB4-genB3-CK1 and CK2).



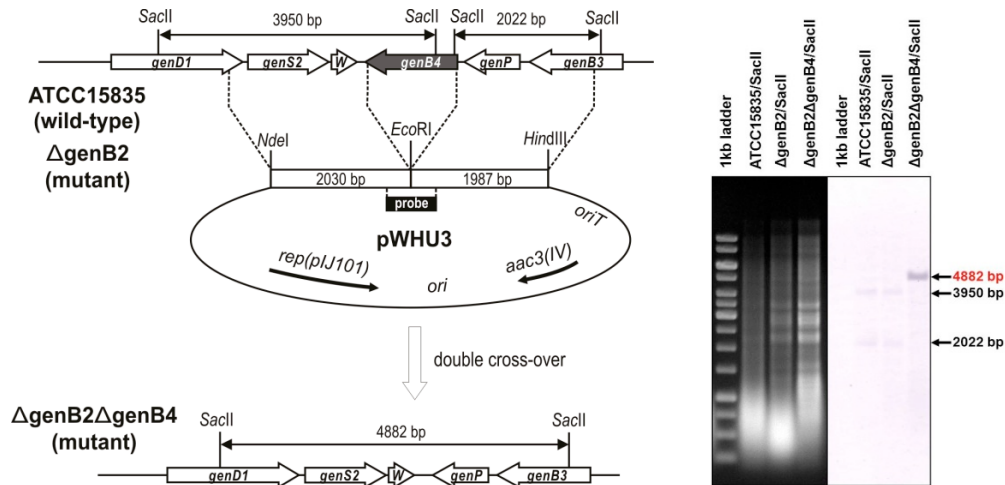
### (8) $\Delta$ genB2 $\Delta$ genB3

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *Psh*AI with a 658 bp probe (PCR-amplified from pWHU5 using oligonucleotide primers genB3-CK1 and CK2).



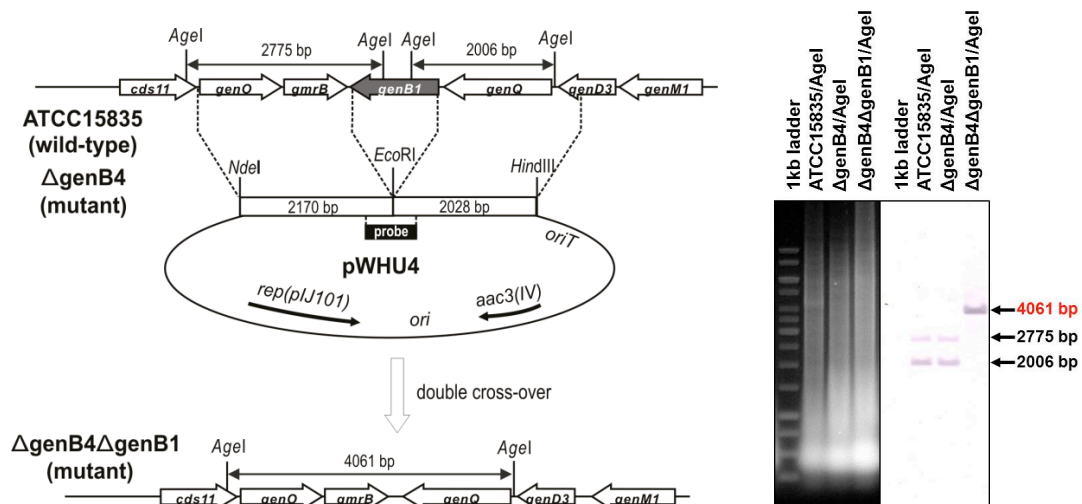
### (9) $\Delta$ genB2 $\Delta$ genB4

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *Sac*II with a 595 bp probe (PCR-amplified from pWHU3 using oligonucleotide primers genB4-CK1 and CK2).



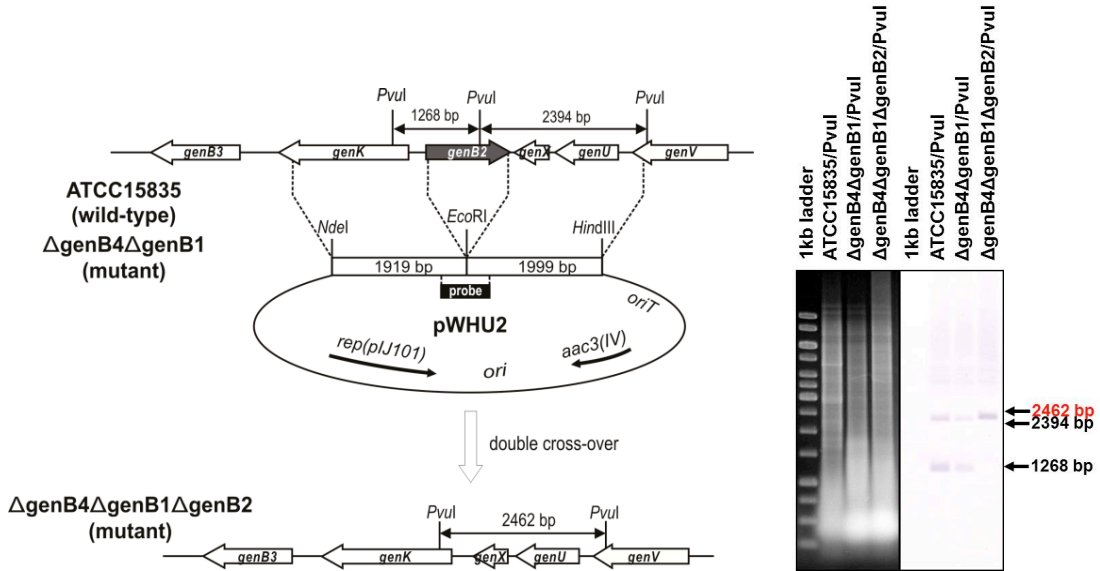
### (10) $\Delta$ genB4 $\Delta$ genB1

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *Age*I with a 581 bp probe (PCR-amplified from pWHU4 using oligonucleotide primers genB1-CK1 and CK2) respectively,.



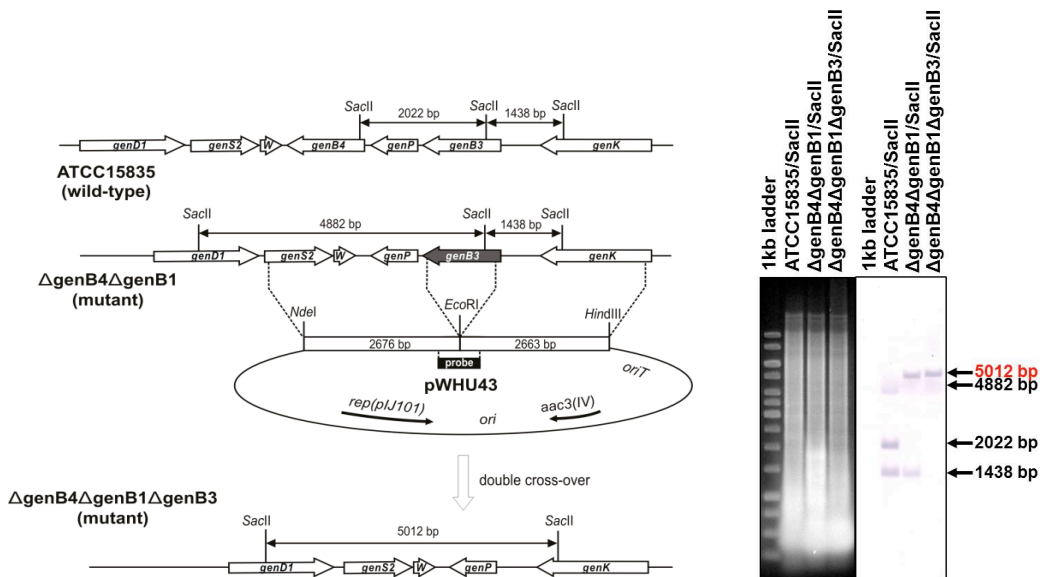
### (11) $\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB2

The arrows indicate the expected size of the fragments from the wild type and mutant chromosomal DNA, respectively, hybridized after digestion with *Pvu*I with a 587 bp probe (PCR-amplified from pWHU2 using oligonucleotide primers genB2-CK1 and CK2).



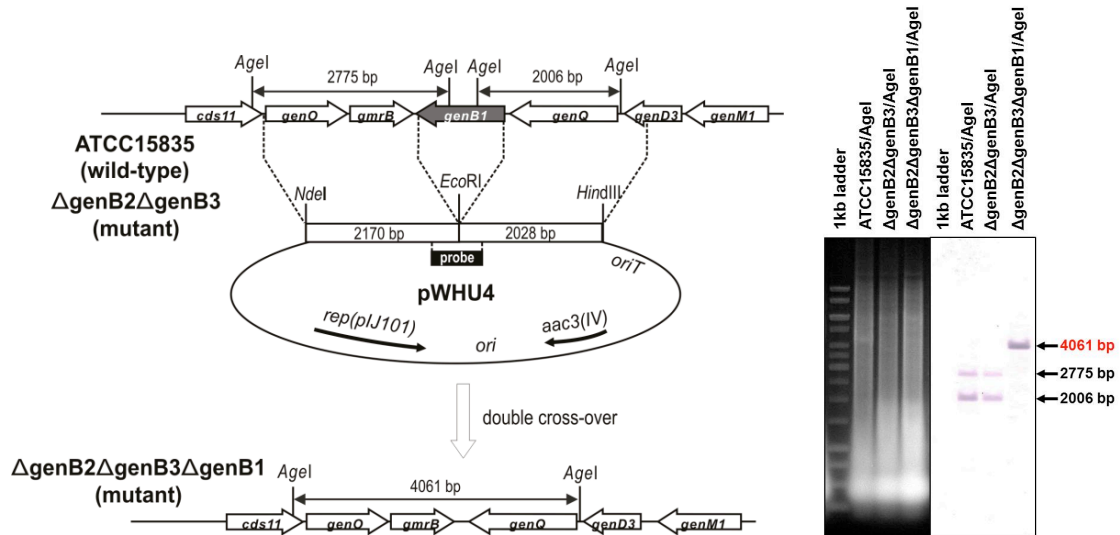
### (12) $\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB3

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *Sac*II with a 741 bp probe (PCR-amplified from pWHU43 using oligonucleotide primers genB4-genB3-CK1 and CK2).



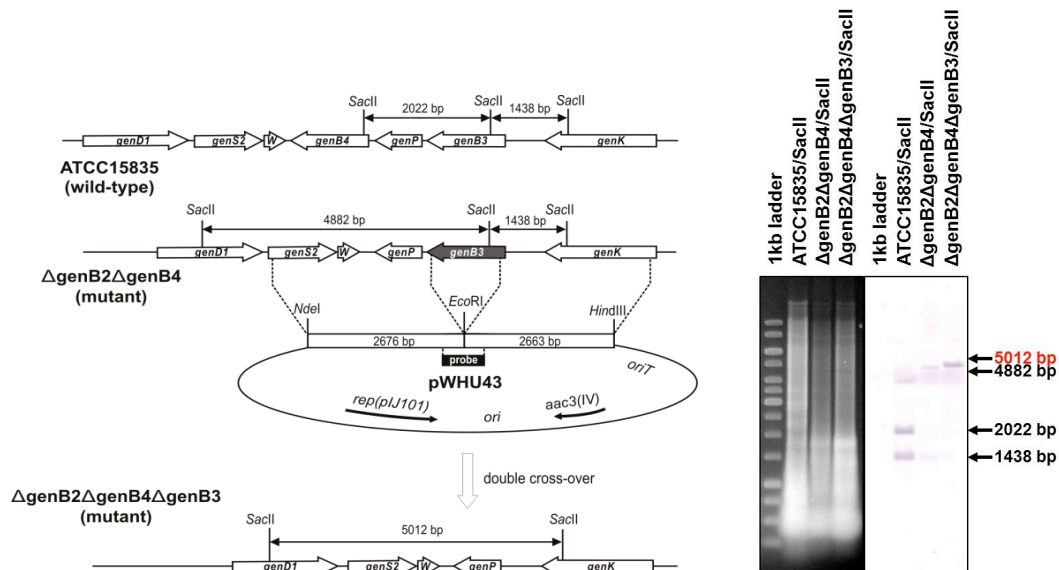
### (13) $\Delta$ genB2 $\Delta$ genB3 $\Delta$ genB1

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *AgeI* with a 581 bp probe (PCR-amplified from pWHU4 using oligonucleotide primers genB1-CK1 and CK2).



### (14) $\Delta$ genB2 $\Delta$ genB4 $\Delta$ genB3

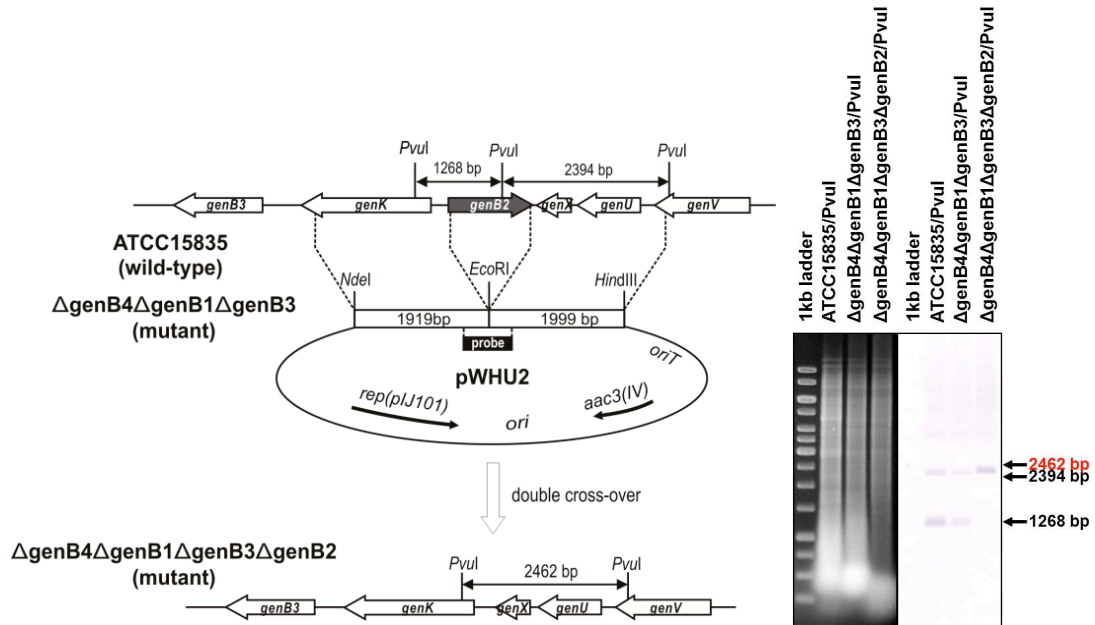
The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *SacI* with a 741 bp probe (PCR-amplified from pWHU43 using oligonucleotide primers genB4-genB3-CK1 and CK2).





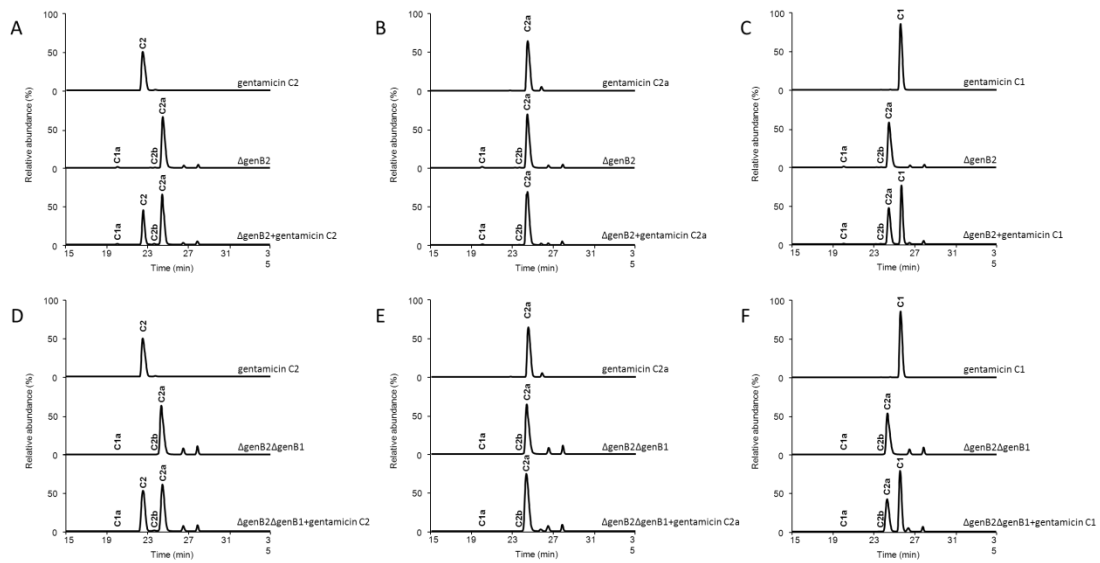
### (15) $\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB3 $\Delta$ genB2

The arrows indicate the expected size of the fragments from the wild type and mutants chromosomal DNA, respectively, hybridized after digestion with *Pvu*I with a 587 bp probe (PCR-amplified from pWHU2 using oligonucleotide primers genB2-CK1 and CK2).



**Figure S5, related to Figure 3. Confirmation of C2a production in  $\Delta$ genB2 and  $\Delta$ genB2 $\Delta$ genB1 mutants**

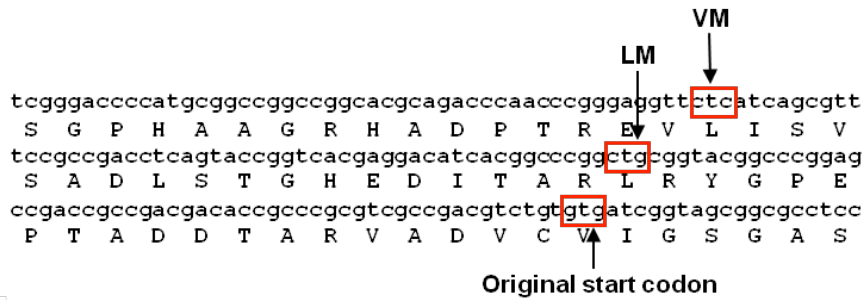
(A) Comparison of product mixture from strain  $\Delta$ genB2 with gentamicin C2 standard; (B) Comparison of product mixture from strain  $\Delta$ genB2 $\Delta$ genB1 with gentamicin C2 standard; (C) Comparison of product mixture from strain  $\Delta$ genB2 with gentamicin C2a standard; (B) Comparison of product mixture from strain  $\Delta$ genB2 $\Delta$ genB1 with gentamicin C2a standard; (E) Comparison of product mixture from strain  $\Delta$ genB2 with gentamicin C1 standard; (F) Comparison of product mixture from strain  $\Delta$ genB2 $\Delta$ genB1 with gentamicin C1 standard.



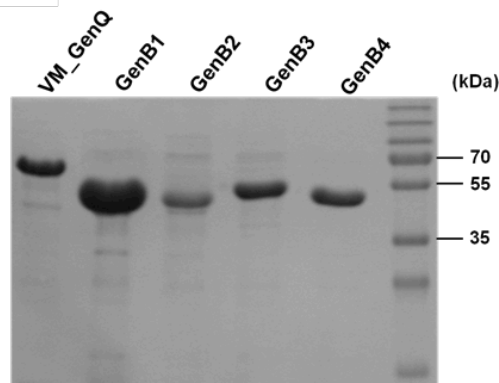
**Figure S6, related to Figures 4, 5 and 6. Cloning and characterization of GenQ, GenB1, GenB2, GenB3 and GenB4**

(A) Cloning *genQ* with alternative start codons. Positions of the original start codon of *genQ* and the new start codons in LM\_ *genQ* and VM\_ *genQ* are indicated in red boxes; (B) SDS-PAGE gel of VM\_ GenQ, GenB1, GenB2, GenB3 and GenB4; (C) UV-Vis spectra of VM\_ GenQ and FAD standard; (D) UV-Vis spectra of GenB proteins.

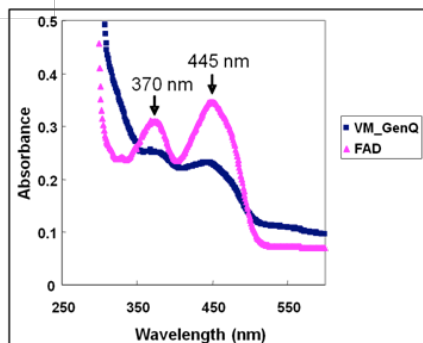
**A**



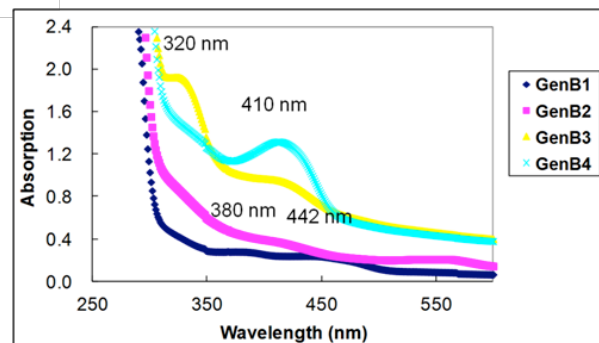
**B**



**C**



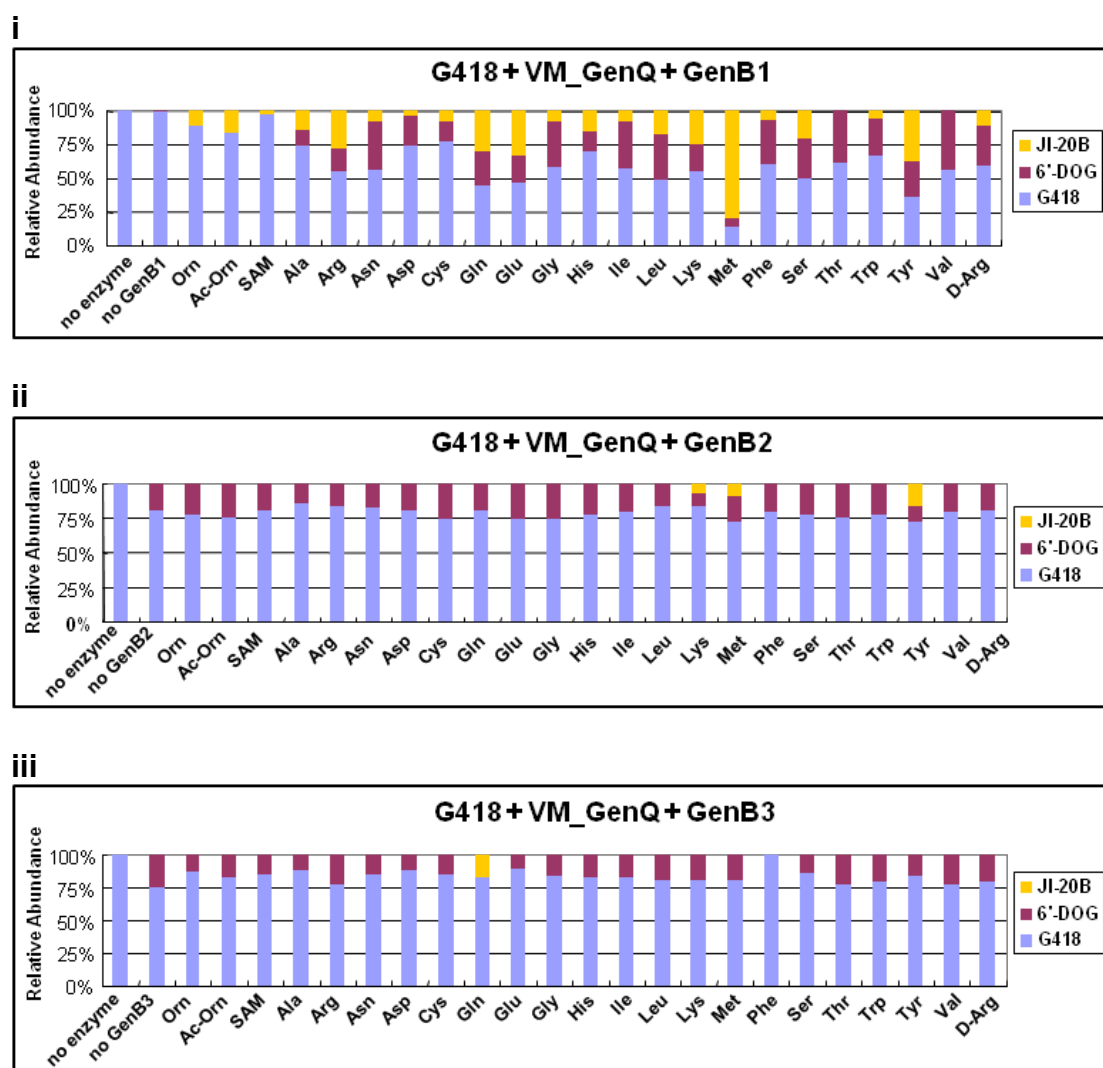
**D**



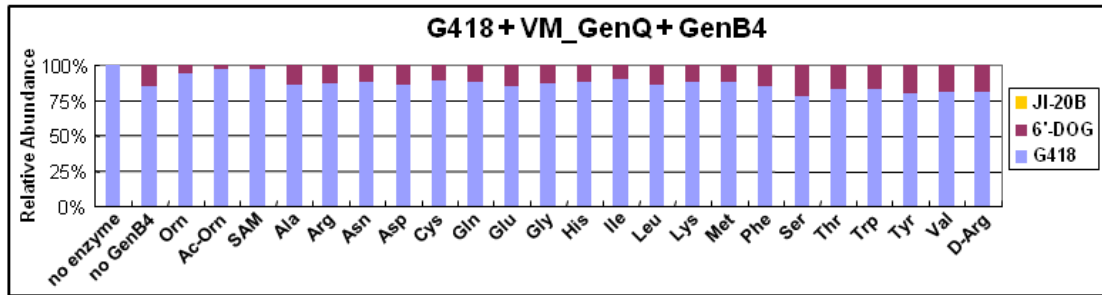
**Figure S7, related to Figures 4 and 5. LC-MS analysis of activity assays of GenB1, GenB2, GenB3 and GenB4 (GenB)**

(A) Test of different amino donors on the conversion of G418 to JI-20B (i - iv) and of gentamicin X2 to JI-20A(v - viii) catalysed by VM\_GenQ coupled with GenB1, GenB2, GenB3, and GenB4 by LC-MS analysis. The percentages of G418, gentamicin X2, 6'-DOG, 6'-DOX, JI-20B and JI-20A present after 16 h of incubation, based on the abundance of corresponding ions detected, are shown as stacked plots. (B) LC-MS analysis of isomerase activity of GenB enzymes on gentamicin C2 and C2a. Selective ion monitoring  $[M+H]^+$  ( $m/z$  464) and  $[M+Na]^+$  ( $m/z$  486) ions of C2 and C2a.

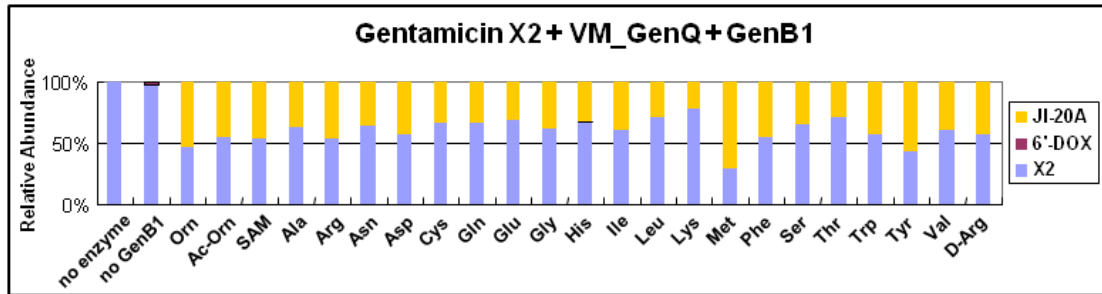
**A**



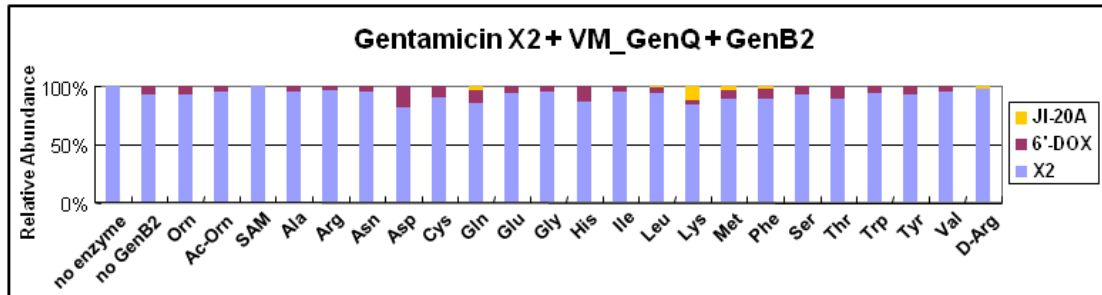
iv



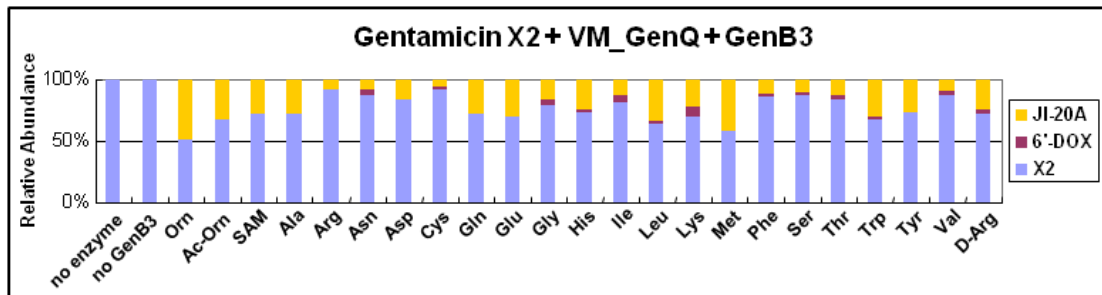
v



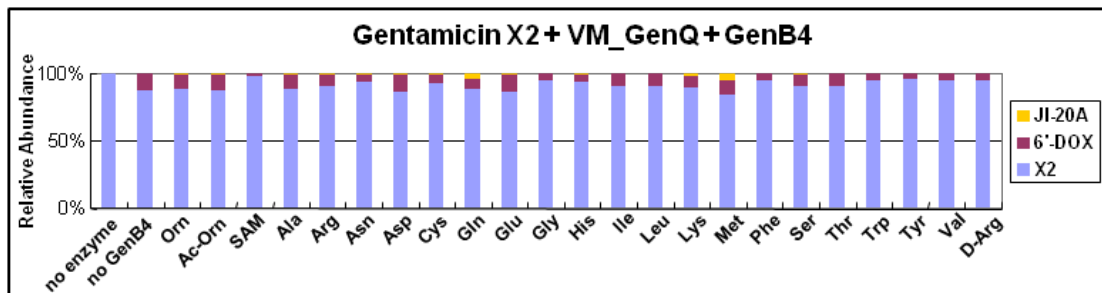
vi



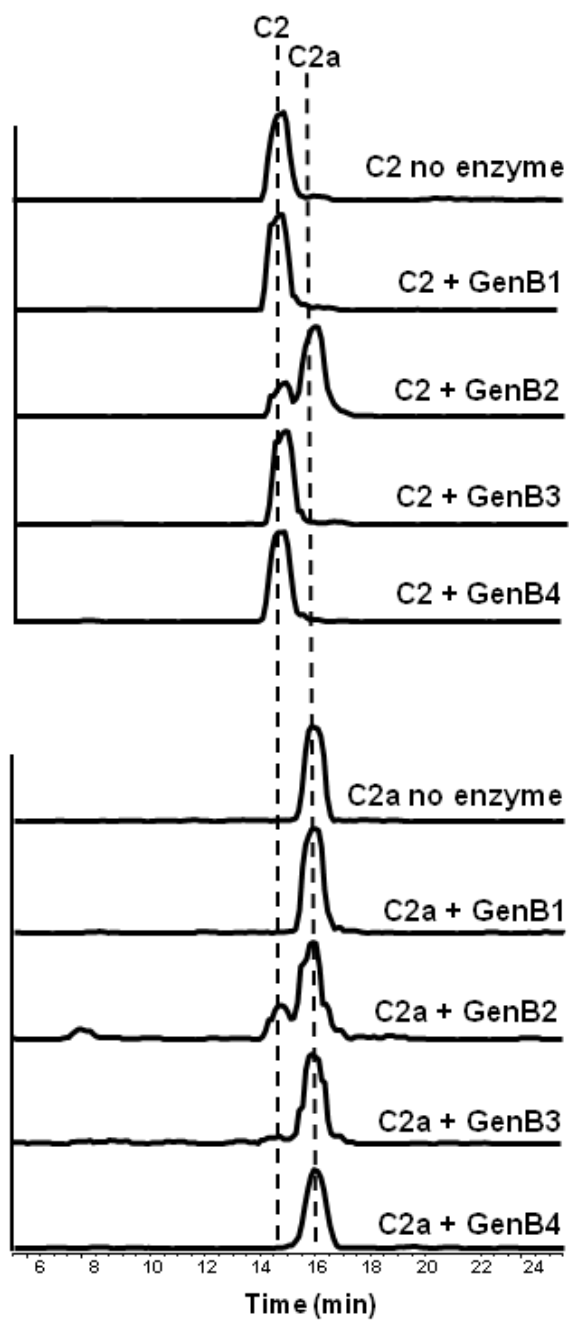
vii



viii



B



**List of oligonucleotide primers used in this study, related to Experimental Procedures**

<b>Primer</b>	<b>Oligonucleotide sequences (5' to 3')</b>	<b>Restriction site</b>
genQ-L1	CTG <b>CATATG</b> CTCACCGCCCGGC	<i>NdeI</i>
genQ-L2	CCC <b>GAATTC</b> TCCCTGTGACCTGTCTG	<i>EcoRI</i>
genQ-R1	ACC <b>GAATTC</b> GCCGCTACCGATCACAC	<i>EcoRI</i>
genQ-R2	GGC <b>AAGCTT</b> GATCGGAACCATCCGG	<i>HindIII</i>
genQ-CP1	CCTCCTCGTCACCGTGG	-
genQ-CP2	CAGGTGCTCAGCGTCCG	-
genQ-EP1	GT <b>CATATG</b> ATCGGTAGCGGCGCC	<i>NdeI</i>
genQ-EP2	CG <b>GAATTC</b> ACAGGGAGAAATCGG	<i>EcoRI</i>
neo11-EP1	CGC <b>CATATG</b> AAGCGCCTTCGAGGC	<i>NdeI</i>
neo11-EP2	CCG <b>GAATTC</b> TCAGACGTGCGCGGTGTG	<i>EcoRI</i>
genK-L1	GCG <b>CATATG</b> AGAACCGGCTTGATGC	<i>NdeI</i>
genK-L2	GCG <b>GAATTC</b> CACTGACCCTCGTCCC	<i>EcoRI</i>
genK-R1	GAG <b>GAATTC</b> CTTCACTTGATTACC	<i>EcoRI</i>
genK-R2	CAC <b>AAGCTT</b> CACCGGCGAGACG	<i>HindIII</i>
genK-CP1	CGGGCGAACCTTCGGGATA	-
genK-CP2	CCGTCAGCGTTGGCAATA	-
genK-EP1	CGC <b>CATATG</b> AACGCGCTGGTGGCA	<i>NdeI</i>
genK-EP2	CCG <b>GAATTC</b> TCAGTGGGAAACCGCCTC	<i>EcoRI</i>
genB1-L1	GCA <b>CATATG</b> CTGGAGAATCCGTCTG	<i>NdeI</i>
genB1-L2	GTC <b>GAATTC</b> CTCTGGGAGGACTGAG	<i>EcoRI</i>
genB1-R1	ACC <b>GAATTC</b> GATGGTCATCGTGTCTG	<i>EcoRI</i>
genB1-R2	GAA <b>AAGCTT</b> GACCGTCAACCTGGCG	<i>HindIII</i>
genB1-CK1	GAGTCGTCGTACTTCTGGATGGG	-
genB1-CK2	GCTGACCTGGGGTTGCTGC	-
genB2-L1	GCG <b>CATATG</b> TCACCGGCGAAGCGGC	<i>NdeI</i>
genB2-L2	CGT <b>GAATTC</b> GTCAGCGTTGGCAATA	<i>EcoRI</i>
genB2-R1	GTG <b>GAATTC</b> GTGACCGCTCAGCTCT	<i>EcoRI</i>
genB2-R2	GTC <b>AAGCTT</b> GTTGCAGGCGGTCCAG	<i>HindIII</i>
genB2-CK1	GCCGACTTCGACCTCTTCC	-
genB2-CK2	ATCCGCTGACCCCGTGCC	-

genB3-L1	CGT <b>CATATG</b> GCAACACCACGTCCG	<i>NdeI</i>
genB3-L2	GAC <b>GAATTC</b> ATCGAGAAGGTGGTC	<i>EcoRI</i>
genB3-R1	CGA <b>GAATTC</b> GGTCCCGATGTCGTAG	<i>EcoRI</i>
genB3-R2	CTT <b>AAGCTT</b> TGGCGAGGGGCTCTGC	<i>HindIII</i>
genB3-CK1	CGCGTTACGGAAAGTAAAATCAC	-
genB3-CK2	CATCGAGGGCCACCACC	-
genB4-L1	CGC <b>CATATG</b> CAGCGGTTCAAGGGCG	<i>NdeI</i>
genB4-L2	GTC <b>GAATTC</b> CCGGCACAGAACTGAC	<i>EcoRI</i>
genB4-R1	GAT <b>GAATTC</b> ACGGTAGTTCATGTGC	<i>EcoRI</i>
genB4-R2	CGG <b>AAGCTT</b> TGTGTGAGCGGTACGG	<i>HindIII</i>
genB4-CK1	TGACTTCTGCCTCGACAACG	-
genB4-CK2	AAGCTCTACCTGGAGACCTTCC	-
genB4-genB3-L1	GGC <b>CATATG</b> CGGTGGCGACCC	<i>NdeI</i>
genB4-genB3-L2	CGC <b>GGTACC</b> AAGCCGGTTCTCA	<i>KpnI</i>
genB4-genB3-R1	CAA <b>GGTACC</b> AGAATCCATGTCC	<i>KpnI</i>
genB4-genB3-R2	AAT <b>AAGCTT</b> GGGAATTAGTCCAG	<i>HindIII</i>
genB4-genB3-CK1	CAGCGTTCCTCCGGTTTC	-
genB4-genB3-CK2	GGTAAGTGATCCGCAGTTGG	-
pGenQ-LM_up	ATCACGGCC <b>CATATG</b> CGGTACGGCCCGGAG	<i>NdeI</i>
pGenQ-VM_up	CCCAACCCGG <b>CATATG</b> CTCATCAGCGTTTC	<i>NdeI</i>
pGenQ-dn	TTGCCGGCACCC <b>GAATTC</b> GATGGTCATCGTG	<i>EcoRI</i>
pGenB1-up	AATCGAGCGAC <b>CATATG</b> ACCATCGACATCG	<i>NdeI</i>
pGenB1-dn	AGGACCTGGTGG <b>GGATCC</b> ACAAGGAGTGAA	<i>BamHI</i>
pGenB2-up	CGGAGGTTTCG <b>CATATG</b> ATTATTGCCAACG	<i>NdeI</i>
pGenB2-dn	CGGCCCTGCC <b>GGATCC</b> GTCAGAGCTGAGC	<i>BamHI</i>
pGenB3-up	TTG GGGATAG <b>CATATG</b> GCAGTCGCCGACCA	<i>NdeI</i>
pGenB3-dn	ATCTTCACCGGCCCTG <b>GAATTC</b> CTACGCC A	<i>EcoRI</i>
pGenB4-up	CGTGGTGGG <b>CATATG</b> AACTACCGTGAGTTG	<i>NdeI</i>
pGenB4-dn	CGGCCCTCGAACCG <b>GAATTC</b> CTCAAGGTCA	<i>EcoRI</i>

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Primer pairs for amplification of left- or right-flanking fragments of a target gene, for PCR/sequencing confirmation, and for cloning target genes for over-expression are marked with suffixes -L1/-L2, -R1/-R2, CK1/CK2, or -up/-dn, respectively.

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**Table S1, related to Figure 3. LC-HRMS analysis of *genB* knock-out mutants**

Strain	Gentamicin C complex production ( $\times 10^5$ )					Intermediates production ( $\times 10^5$ )			
	C1a	C2b	C2	C2a	C1	X2	JI-20A	G418	JI-20B
wild type	8.8	2.8	11.9	1.1	44.6	8.5	1.5	8.2	4.3
$\Delta$ genB1	0.4	2.9	1.2	1.7	14.6	6.8	0.5	15.9	2.5
$\Delta$ genB2	0.8	0.1	ND	7.6	ND	7.1	0.7	0.7	1.0
$\Delta$ genB3	ND	ND	ND	ND	ND	7.2	19.1	10.8	75.4
$\Delta$ genB4	ND	ND	ND	ND	ND	11.0	2.7	14.1	8.7
$\Delta$ genB2 $\Delta$ genB3	ND	ND	ND	ND	ND	8.6	2.7	8.8	20.5
$\Delta$ genB2 $\Delta$ genB4	ND	ND	ND	ND	ND	7.6	2.0	1.2	3.7
$\Delta$ genB4 $\Delta$ genB1	ND	ND	ND	ND	ND	5.2	1.4	18.6	6.7
$\Delta$ genB2 $\Delta$ genB1	0.3	0.1	ND	15.3	ND	4.0	0.5	10.7	2.9
$\Delta$ genB1 $\Delta$ genB3	ND	ND	ND	ND	ND	1.5	0.1	43.0	1.4
$\Delta$ genB4 $\Delta$ genB3	ND	ND	ND	ND	ND	9.7	1.1	17.0	6.3
$\Delta$ genB2 $\Delta$ genB3 $\Delta$ genB1	ND	ND	ND	ND	ND	3.4	0.1	12.8	0.1
$\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB2	ND	ND	ND	ND	ND	1.9	0.1	40.3	3.4
$\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB3	ND	ND	ND	ND	ND	3.3	0.1	20.8	0.8
$\Delta$ genB2 $\Delta$ genB4 $\Delta$ genB3	ND	ND	ND	ND	ND	7.4	2.5	20.8	35.2
$\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB3 $\Delta$ genB2	ND	ND	ND	ND	ND	2.3	ND	142.0	ND

**Table S2, related to Experimental Procedures. Plasmids used in this study**

Plasmid	Description	Reference
pUC18	Sub-cloning vector	Sambrook and Maniatis
pET28a(+)	Vector for protein expression	Invitrogen
pIB139	Site-specific integrative vector with <i>PermE*</i> promoter	Wilkinson et al., 2002; Del Vecchio et al., 2003
pYH7	<i>E. coil</i> - <i>Streptomyces</i> shuttle vector	Sun et al., 2008
pWHU77	pIB139 derivative with Tsr <sup>R</sup>	This study
pYH286	<i>genQ</i> in-frame deletion construct	This study
pWHU1	<i>genK</i> in-frame deletion construct	This study
pWHU67	$\Delta$ <i>genK</i> complementation construct containing <i>genK</i> under control of <i>PermE*</i> promoter	This study
pWHU163	$\Delta$ <i>genQ</i> complementation construct containing <i>genQ</i> under control of <i>PermE*</i> promoter	This study
pWHU165	$\Delta$ <i>genQ</i> complementation construct containing <i>neo11</i> under the control of the <i>PermE*</i> promoter	This study
pWHU4	<i>genB1</i> in-frame deletion construct	This study
pWHU2	<i>genB2</i> in-frame deletion construct	This study
pWHU5	<i>genB3</i> in-frame deletion construct	This study
pWHU3	<i>genB4</i> in-frame deletion construct	This study
pWHU43	<i>genB3</i> and <i>genB4</i> double in-frame deletion construct	This study
pET28/LM_genQ	pET28a(+) vector with VM_ <i>genQ</i> gene insert	This study
pET28/VM_genQ	pET28a(+) vector with LM_ <i>genQ</i> gene insert	This study
pET28/genB1	pET28a(+) vector with <i>genB1</i> gene insert	This study
pET28/genB2	pET28a(+) vector with <i>genB2</i> gene insert	This study
pET28/genB3	pET28a(+) vector with <i>genB3</i> gene insert	This study
pET28/genB4	pET28a(+) vector with <i>genB4</i> gene insert	This study

**Table S3, related to Experimental Procedures. In-frame deletion mutants and complemented strains used in this study**

<b>Gene knockout mutant</b>	<b>Parent strain</b>	<b>Plasmid used to achieve deletion</b>
$\Delta$ genK	wild type	pWHU1
$\Delta$ genQ	wild type	pYH286
$\Delta$ genQ $\Delta$ genK	$\Delta$ genQ	pWHU1
$\Delta$ genB1	wild type	pWHU4
$\Delta$ genB2	wild type	pWHU2
$\Delta$ genB3	wild type	pWHU5
$\Delta$ genB4	wild type	pWHU3
$\Delta$ genB2 $\Delta$ genB1	$\Delta$ genB2	pWHU4
$\Delta$ genB1 $\Delta$ genB3	$\Delta$ genB1	pWHU5
$\Delta$ genB4 $\Delta$ genB3	$\Delta$ genB4	pWHU43
$\Delta$ genB2 $\Delta$ genB3	$\Delta$ genB2	pWHU5
$\Delta$ genB2 $\Delta$ genB4	$\Delta$ genB2	pWHU3
$\Delta$ genB4 $\Delta$ genB1	$\Delta$ genB4	pWHU4
$\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB2	$\Delta$ genB4 $\Delta$ genB1	pWHU2
$\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB3	$\Delta$ genB4 $\Delta$ genB1	pWHU43
$\Delta$ genB2 $\Delta$ genB3 $\Delta$ genB1	$\Delta$ genB2 $\Delta$ genB3	pWHU4
$\Delta$ genB2 $\Delta$ genB4 $\Delta$ genB3	$\Delta$ genB2 $\Delta$ genB4	pWHU43
$\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB3 $\Delta$ genB2	$\Delta$ genB4 $\Delta$ genB1 $\Delta$ genB3	pWHU2
<b>Complemented mutant</b>	<b>Parent strain</b>	<b>Plasmid for complementation</b>
$\Delta$ genK:: <i>genK</i>	$\Delta$ genK	<i>genK</i> gene in pWHU77
$\Delta$ genQ:: <i>genQ</i>	$\Delta$ genQ	<i>genQ</i> gene in pWHU77
$\Delta$ genQ:: <i>neo11</i>	$\Delta$ genQ	<i>neo11</i> gene in pWHU77