Table S1: Strains used in this study

Table S2: Plasmids used in this study

Table S3: Oligonucleotides used in this study

Fig S1. Complementation constructs for *ligC1* and *ligC2* and *ligCTB*. LigC1 and LigC2 are divergently transcribed in the *M. smegmatis* genome. The active site lysine for LigC1 (K32) and LigC2 (K29) are indicated. The complementation constructs for LigC1, LigC1-HA, LigC2, LigC2 HA, MOP-LigC2-HA are indicated as horizontal lines.

Fig S2. Amplification products from white colonies of *ligD*-(K484A) and *ligD*-(D136A-D138A) strains in the I-SceI chromosomal assay. Representative gels showing the PCR-amplification products of the white colonies from *ligD*-(K484A) (A) and *ligD*-(D136A-D138A) (B) strains, obtained using primers that anneal 836 bp upstream and 511 bp downstream from the first I-SceI site on the chromosome. The PCR product arising from amplification of an intact chromosomal I-SceI site is indicated by the 1350 bp band (denoted by an arrow next to the gel) and is marked as “I” above the lane. NHEJ events with a deletion are marked as “D” above the lane.

Fig S3. Blunt-end junction sequences of non-faithful repair events in the strains *ligD*-(K484A) and *ligD*-(K484A)Δ*ligC1/C2/B* complemented with *ligC1*. The two halves of the EcoRV recognition site are colored blue and red. Non-templated nucleotide additions are shown in green. The length of the deletion is indicated by a number next to the junction.

Fig S4. Junction sequences of non-faithful repair events at the 5′ end in the strains *ligD*-(K484A) and *ligD*-(K484A)Δ*ligC1/C2/B* complemented with *ligC1*. The two halves of the Asp718I recognition site are shown in blue and red. Templated and non-templated nucleotide additions are shown in green. The length of the deletion is indicated by a number next to the junction.

Fig S5. Amino acid sequence alignment of LigC1, LigCTB and LigC2. Clustal W2 was used to align amino acid sequences of LigC1, LigC2 and LigCTB. Amino acid sequence identity is marked by an asterisk (*), a match of the functional group by a colon (:) and a match of the charge of the R group by a full stop (.).

Fig S6. Relative mRNA expression level of *ligC1* (A), *ligC2* (B) and *ligCTB* (C) in complementation strains. The level of *ligC1*, *ligC2* and *ligCTB* mRNA expressed from *ligC1*, *MOP* and *ligC2* promoters was measured using RT-PCR in strains complemented with *ligC1*, *ligC2* and *ligCTB.*

Fig S7. Ku is upregulated in nonreplicating *M. smegmatis*. RT-qPCR quantitation of *ku* (A), *ligD* (B), *ligC1* (C) and *ligC2* (D) in log and stationary phases. Relative mRNA levels are given in relation to the mRNA for *sigA*. E) β-galactosidase activity measured using the fluorescent β-galactosidase substrate C2FDG and reported as relative fluorescent units (RFU) at different cell densities. Grey bars are the control strain containing a promoterless *lacZ*. Significant differences ($p<0.05$) in mRNA expression or promoter activity are marked with an asterisk (*).

Table S1

Strain	**Genotype**	**Reference**	**Description**
Wild-type	*M. smegmatis* MC^{2}155		
MGM 139	Δ*ligC1-C2*	(1)	
MGM 140	Δ*ligD*	(1)	
MGM 154	Δ*ku*	(1)	
MGM 801	*ligD*-(D136A-D138A)	(2)	inactivated polymerase
MGM 802	*ligD*-(ΔPOL)	(2)	POL domain deletion
MGM 803	*ligD*-(K484A)	(3)	inactivated ligase
MGM 805	*ligD*-(ΔLIG)	(2)	LIG domain deletion
MGM 806	*ligD*-(E310A)	(2)	no phosphomonoesterase activity
MGM 807	*ligD*-(H336A)	(2)	no phosphomonoesterase and phosphodiesterase activities
MGM 808	Δ*ligB/*Δ*ligC/*Δ*ligD*	(2)	Lacking all ATP dependent DNA ligases
MGM 810	*ligD*-(K484A)Δ*ligC1-C2/*Δ*ligB*	(2)	
MGM 812	*ligD*-(POL only)	This work	
MGM 832h	MGM 810::pDB60	This work	*ligD*-(K484A) Δ*ligC1-C2/B* + vector
MGM 833h	MGM 810::pHB3	This work	*ligD*-(K484A) Δ*ligC1-C2/B* +LigB
MGM 834h	wild-type::pDB60	This work	WT + vector
MGM 835h	MGM 803::pDB60	This work	*ligD*-(K484A)+vector
MGM 839h	MGM 810::pHB1	This work	*ligD*-(K484A) Δ*ligC1-C2/B* +LigC1
MGM 840h	MGM 810::pHB2	This work	*ligD*-(K484A) Δ*ligC1-C2/B* +LigC2
MGM 843	Δ*polD1*	(4)	
MGM 844h	Δ*polD1* in MGM 803	This work	
MGM 846	*ligD*-(D136A-D138A)Δ*polD1-D2*	(4)	
MGM 854h	MGM 801::pDB60	This work	LigD-POL dead +vector
MGM 856h	MGM 154::pDB60	This work	Δ*ku* + vector
MGM 858h	MGM 140::pDB60	This work	Δ*ligD* + vector

MGM 859h	MGM 808::pDB60	This work	Δ*ligB/*Δ*ligC/*Δ*ligD* + vector
MGM 860h	MGM 808::pHB1	This work	Δ*ligB/*Δ*ligC/*Δ*ligD* +LigC1
MGM 861h	MGM 808::pHB2	This work	Δ*ligB/*Δ*ligC/*Δ*ligD* +LigC2
MGM 863h	MGM 810::pHB6	This work	*ligD*-(K484A)Δ*ligC1-C2/*Δ*ligB+*LigC2-HA
MGM 864h	MGM 810::pHB7	This work	*ligD*-(K484A)Δ*ligC1-C2/*Δ*ligB+*MOP-LigC2-HA
MGM 865h	MGM 810::pHB5	This work	*ligD*-(K484A)Δ*ligC1-C2/*Δ*ligB+*LigC1-HA
MGM 866h	MGM 810::pHB8	This work	*ligD*-(K484A)Δ*ligC1-C2/*Δ*ligB+*LigCTB
MGM 867h	wild-type::pJEM 13	This work	promoterless *lacZ*
MGM 164h	wild-type::pMSG335	This work	*lacZ* under native *ku* promoter
Mgm1602	mc^{2}155 *attB*::pRGM10	(5)	
Mgm1605	Mgm140 *attB*::pRGM10	(5)	
Mgm1642	Mgm801 *attB*::pRGM10	This work	
Mgm1644	Mgm802 *attB*::pRGM10	This work	
Mgm1638	Mgm803 *attB*::pRGM10	This work	
Mgm1646	Mgm805 *attB*::pRGM10	This work	

1. **Gong C, Bongiorno P, Martins A, Stephanou NC, Zhu H, Shuman S, Glickman MS**. 2005. Mechanism of nonhomologous end-joining in mycobacteria: a low-fidelity repair system driven by Ku, ligase D and ligase C. Nat Struct Mol Biol **12**:304–312.

2. **Aniukwu J, Glickman MS, Shuman S**. 2008. The pathways and outcomes of mycobacterial NHEJ depend on the structure of the broken DNA ends. Genes Dev **22**:512–527.

3. **Akey D, Martins A, Aniukwu J, Glickman MS, Shuman S, Berger JM**. 2006. Crystal structure and nonhomologous end-joining function of the ligase component of Mycobacterium DNA ligase D. J Biol Chem **281**:13412–13423.

4. **Zhu H, Bhattarai H, Yan HG, Shuman S, Glickman MS**. 2012. Characterization of Mycobacterium smegmatis PolD2 and PolD1 as RNA/DNA polymerases homologous to the POL domain of bacterial DNA ligase D. Biochemistry **51**:10147–10158.

5. **Gupta R, Barkan D, Redelman-Sidi G, Shuman S, Glickman MS**. 2011. Mycobacteria exploit three genetically distinct DNA double-strand break repair pathways. Mol Microbiol **79**:316–330.

Table S2

Plasmid name	**Relevant feature**	**Source**
pHB1	pDB60 *nat_ligC1*	This study
pHB2	pDB60 *nat_ligC2*	This study
pHB3	pDB60 *nat_ligB*	This study
pHB4	pDB60MOP_ligC2	This study
pHB5	pDB60 *nat_ligC1HA*	This study
pHB6	pDB60 *nat_ligC2HA*	This study
pHB7	pDB60 *MOP_ligC2HA*	This study
pHB8	pDB60 *ligC1p_ligCTBHA*	This study
pJEM13	lacZ translational fusion vector	(1)
pMSG335	Translational fusion of Ku (AA1-4) to B galactosidase	This study
pDB60	*attB* site and L1 transposon	(2)
pRGM10	I-SceI recombination substrate to assay NHEJ, SSA and HR	(3)

1. **Timm J, Lim EM, Gicquel B**. 1994. Escherichia coli-mycobacteria shuttle vectors for operon and gene fusions to lacZ: the pJEM series. J. Bacteriol. **176**:6749–53.

2. **Barkan D, Liu Z, Sacchettini JC, Glickman MS**. 2009. Mycolic acid cyclopropanation is essential for viability, drug resistance, and cell wall integrity of Mycobacterium tuberculosis. Chem. Biol. **16**:499–509.

3. **Gupta R, Barkan D, Redelman-Sidi G, Shuman S, Glickman MS**. 2011. Mycobacteria exploit three genetically distinct DNA double-strand break repair pathways. Mol Microbiol **79**:316–330.

Table S3

oligonucleotides	sequence (5’ to 3’)	note
ligC1_3’	GGATCTGGTTCACGGACTTC	
ligC5’	CGCACTGGCCATCACTGTTC	
ligC3’	ACTGCGGTTCGGCTAAACCC	
ligC2_5’	GCCTTGGCAAGCATCGGTTC	
HA tag to LigC2	CCGATATCTTAAGCGTAGTCTGGGACGTCGTATGGGTAAACCCGGCACGATGTCAC	
HA into LigC1	CCTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTACTGTTCCTCCAGCACGTCG	
LigB_pro_for	ATCAGTCGCGCTCGTAGAAC	
LigB_pro_rev	CTGGCACTACGTCACCTTCG	
ligCTBwithC1pF	GGTAAGAATGGGAAGGATGCAGTTACCCGTC	
ligCTBwithC1pR	GACGGGTAACTGCATCCTTCCCATTCTTACC	
ligCTBHAtag	CCGATATCTTAAGCGTAGTCTGGGACGTCGTATGGGTAGCGTAGGCCCGGCAC	
HB23	TCGAAGAACGCCTCTTCC	*ligD* qPCR
HB24	CCTGACAAGGTGCTCTATCC	*ligD* qPCR
HB17	GCCGGTCAAGGTCTACAG	*ku* qPCR
HB18	GTTGATGTCGCGGTACTCG	*ku* qPCR
HB9	CGAGCAGTTCGGGAAAG	*ligC1* qPCR
HB10	AAGGCGCAGGTGAAG	*ligC1* qPCR
HB11	GTCGCCCATGCTGTC	*ligC2* qPCR
HB12	CGCGGCCACCAATTC	*ligC2* qPCR
HB21	TGGCGATGATGATCTCCC	*ligCTB*(Rv3731) qPCR
HB22	TCCGCTCCATCTGCTTTC	ligCTB(Rv3731) qPCR

oAF485	CCAAGCGGGCAGCCAAG	*sigA* qPCR
oAF486	GGGCTCAGCCTCGAGATCGTC	*sigA* qPCR
ku promoter 5’	TCCGCGGCACATAAGTGGCACGTTC	
ku promoter 3’	TGGTACCGATGGATCGCATAAGGCCAG	

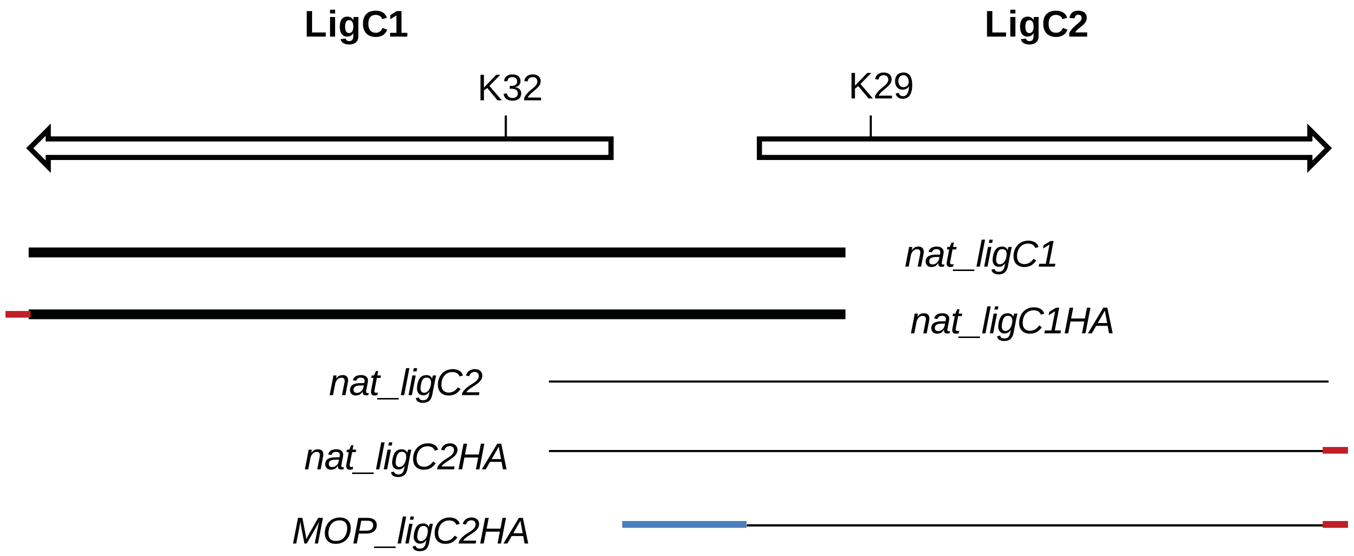
LigC1
LigC2
K32
K29
nat_ligC1
nat_ligC1HA
nat_ligC2
nat_ligC2HA
MOP_ligC2HA

Figure S1

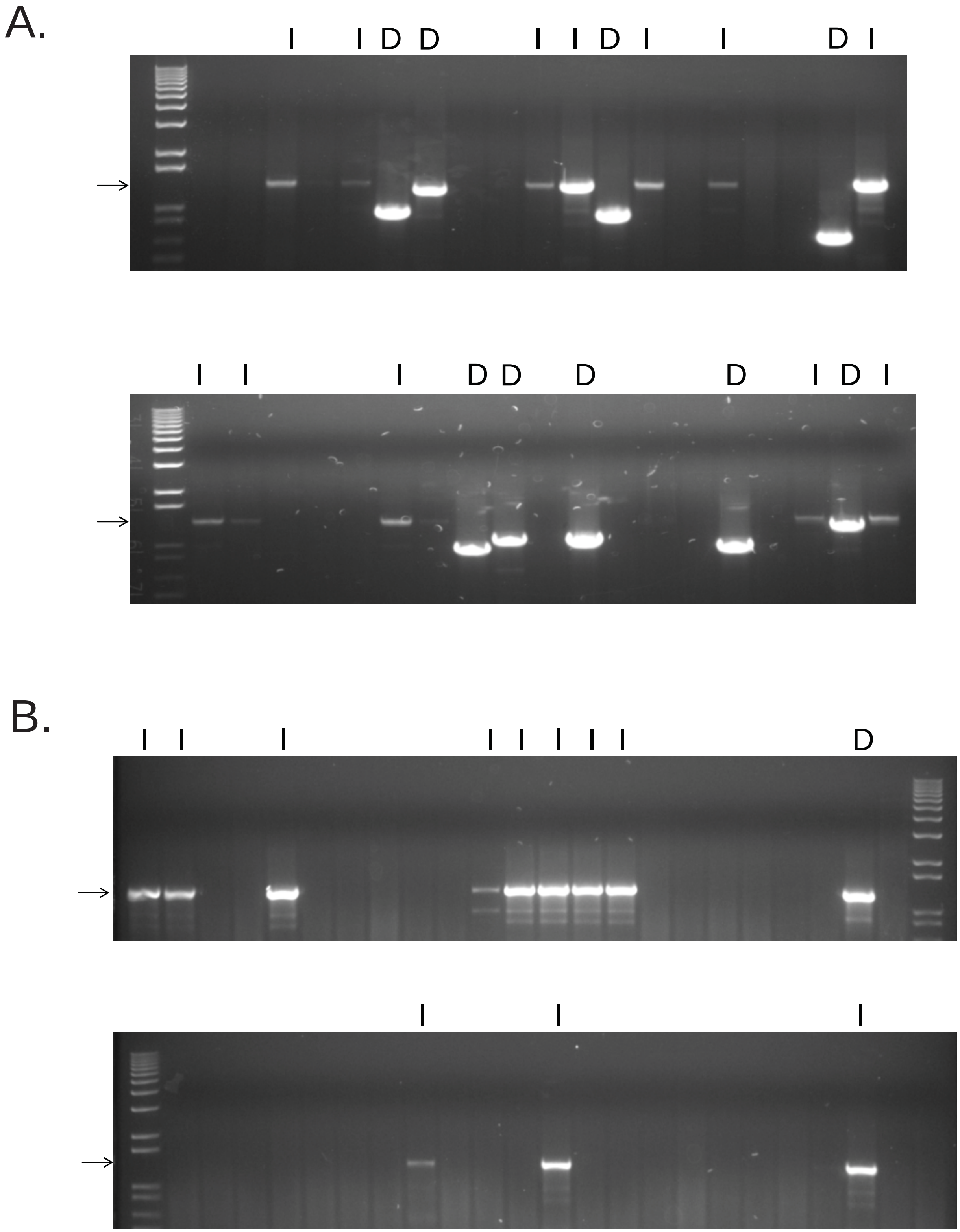

Figure S2

Original

```
GATGGTGCAGGATATCCTGCTGATGA
CTACCACGTCCTATAGGACGACTACT
```

ligD-(K484A) junctions

```
GGTCTGCTGCTGC    -95/-205    TGCAGCGCGATCG
CCAGACGACGACG                ACGTCGCGCTAGC

TGATTGAAGCAGA   -148/-178    CGATGAGCGAACG
ACTAACTTCGTCT                GCTACTCGCTTGC

CGCTCACATTTAA   -742/-353    CACGGCCACCGAT
GCGAGTGTAAATT                GTGCCGGTGGCTA

GATGGTGCAGGATGATCCTGCTGATGA
CTACCACGTCCTACTAGGACGACTACT

GATGGTGCAGGAT       /-562    AGGGCGGCTTCGT
CTACCACGTCCTA                TCCCGCCGAAGCA

GATGGTGCAGGATT      /-678    TGTATGAACGGTC
CTACCACGTCCTAA               ACATACTTGCCAG

GCAGAAGCCTGCG   -140/        ATCCTGCTGATGA
CGTCTTCGGACGC                TAGGACGACTACT

TTTCCGTGACGTC   -489/        ATCCTGCTGATGA
AAAGGCACTGCAG                TAGGACGACTACT

GATGGTGCAGGAT       /-104    GGATGAAGCCAAT
CTACCACGTCCTA                CCTACTTCGGTTA
```

ligD-(K484A))*ΔligB/CattB:nat_ligC1* junctions

```
TGCACACCGCCGA   -169/-170    GCTACCGGCGATG
ACGTGTGGCGGCT                CGATGGCCGCTAC

GATGGTGCAGGATGATCCTGCTGATGA
CTACCACGTCCTACTAGGACGACTACT

GCAGAAGCCTGCG   -140/-129    GGCATGGTGCCAA
CGTCTTCGGACGC                CCGTACCACGGTT

ATCGATGAGCGTG   -278/-328    AGTATGAAGGCGG
TAGCTACTCGCAC                TCATACTTCCGCC

TGATGGTGCTGCG   -556/-37     GCTGTTCGCATTA
ACTACCACGACGC                CGACAAGCGTAAT

TCTATCGTGCGGT   -190/-136    TGCCAATGAATCG
AGATAGCACGCCA                ACGGTTACTTAGC

GATGGTGCAGGAT       /-455    TGGAGAGACGCGC
CTACCACGTCCTA                ACCTCTCTGCGCG

GAAGCAGAAGCCT   -143/-208    AGCGCGATCGTAA
AGATAGCACGCCA                TCGCGCTAGCATT

GTCGAAAACCCGA   -230/-168    TGGCTACCGGCGA
CAGCTTTTGGGCT                ACCGATGGCCGCT

GATGGTGCAGGATCATCCTGCTGATGA
CTACCACGTCCTAGTAGGACGACTACT

GATGGTGCAGGAT       /-206    GCAGCGCGATCGT
CTACCACGTCCTA                CGTCGCGCTAGCA

GGTCTGCTGCTGC    -95/-243    TCGCTGGGGAATG
AGATAGCACGCCA                AGCGACCCCTTAC
```

Figure S3

Original
GAATCTGCATGGTACCAAGCTTGCTC
CTTAGACGTACCATGGTTCGAACGAG

ligD-(K484A) junctions

CTCAATGCCCCCT GAGTTACGGGGGA	-84/-80	CCCCTTTCGCCAG GGGGAAAGCGGTC
TTCACAAAACGGT AAGTGTTTTGCCA	-196/	GTACCAAGCTTGCTC CATGGTTCGAACGAG
GAATCTGCATGGTAC CTTAGACGTACCATG	/-35	GGGAAAACCCTGG CCCTTTTGGGACC
TGATTCGATATTT ACTAAGCTATAAA	-30/-163	CGCTTTGCCTGGT GCGAAACGGACCA
GTGGCCGCTTGCG CACCGGCGAACGC	-47/266	ACGGTTACGATGC TGCCAATGCTACG
CTGACCCCGGGGT GACTGGGGCCCCA	-130/-54	CCAACTTAATCGC GGTTGAATTAGCG
TAACTGTAGGCCT ATTGACATCCGGA	-141/-1216	GTGGATGAAGCCA CACCTACTTCGGT
AGCGGTTAGCTCC TCGCCAATCGAGG	-409/-1245	CATGGTGCCAATG GTACCACGGTTAC

ligD-(K484A))*ΔligB/CattB:nat_ligC1* junctions

CCCCCTAACCCGC GGGGGATTGGGCG	-77/	GTACCAAGCTTGCTC CATGGTTCGAACGAG
TTTTCACAAAACG AAAAGTGTTTTGC	-198/	GTACCAAGCTTGCTC CATGGTTCGAACGAG
TTTTCACAAAACG AAAAGTGTTTTGC	-198/	GTACCAAGCTTGCTC CATGGTTCGAACGAG
GCTAGTACTGGGC CGATCATGACCCG	-170/-74	CACATCCCCCTTT GTGTAGGGGGAAA
GAATCTGCATGGTACA CTTAGACGTACCATGT	/-149	TGAATGGCGAATG ACTTACCGCTTAC
GAATCTGCATGGTAC CTTAGACGTACCATG	/-362	TCACATTTAATGT AGTGTAAATTACA
TAAAGCTAGTACT ATTTCGATCATGA	-174/-266	ACGGTTACGATGC TGCCAATGCTACG
GAATCTGCATGGTACC CTTAGACGTACCATGG	/-227	AGGCCGATACTGT TCCGGCTATGACA
TCTTACTGTCATG AGAATGACAGTAC	-319/-79	CCCCCTTTCGCCA GGGGGAAAGCGGT

Figure S4

```
LigC1           MDLPVQPPIEPMLAKAQVKVPDEAGVWSYEPKWDGFRALVFRDGDDVVLQSRNGKDLGRY 60
LigCTB          MQLPVMPPVSPMLAKSVTAIPPDA---SYEPKWDGFRSICFRDGDQVELGSRNERPMTRY 57
LigC2           MDLPVLPPVSPMLSKSVNQIPPGM---SYEPKWDGFRSILFRDGAEVELGSRKERPMTRY 57
                *:*** **:.***:*:   :*      **********:: **** :* * **: : : **

LigC1           FPELLDALRDELVEKCVLDGEVVVPRDIAGRVRLDWESLSQRIHPAASRIKMLAEQTPAH 120
LigCTB          FPELVAAIRAELPHRCVIDGEIIIATDHG----LDFEALQQRIHPAESRVRMLADRTPAS 113
LigC2           FPELVAAALTELPDRCVIDGEIVLPADNH----LDFEALQLRLHPAASRVAMLAEQTPAA 113
                ****: *   ** .:**:***:::. *      **:*:*. *:*** **: ***::***

LigC1           FIGFDALALGDRSLLKEPFRVRREALAEAVDNKRWC-----HVTRTSEDPALGTEWLKTF 175
LigCTB          FIAFDLLALGDDDYTGRPFSERRAALVDAVTGSGADADLSIHVTPATTDMATAQRWFSEF 173
LigC2           FIAFDLLALGDDDYTGRPFSERRAALETALADAGP----TFHLTPATTDLPTAQRWFHEF 169
                **.** ***** .   .**  ** **  *: .         *:* :: * . . .*:  *

LigC1           EGAGLDGVIAKRLDGPYLPGKREMVKVKHHRDADCVAMGYRIHKSG-DGIGSILLGLYRD 234
LigCTB          EGAGLDGVIAKPPHITYQPDKRVMFKIKHLRTADCVVAGYRVHKSGSDAIGSLLLGLYQE 233
LigC2           EGAGLDGVIAKPLDLTYQPDKRVMFKVKHQRTADCVVAGYRLHKSGADAVGSLLLGLYDD 229
                ***********  . .* *.** *.*:** * ****. ***:**** *.:**:***** :

LigC1           DGELQMVGGAASFTAKDRIKLLAELEPLRE-----------------GDEMREGDPSRWN 277
LigCTB          DGQLASVGVIGAFPMAERRRLLTELQPLVTSFDDHPWNWAAHVAG--QRTPRKNEFSRWN 291
LigC2           DGSLASVGVIGAFPMATRRALFTELQTLVADFDHHPWNWAAQAAADPELARRYGGGSRWN 289
                **.*  **  .:*.   *  *::**:.*                       * .  ****

LigC1           SAADKRWTPLRPEKVCEVAYDQMEGNSVEGRRFRHAVKFLRWRPDREPSSCTFDQLDTPL 337
LigCTB          VGKDLSFVPLRPERVVEVRYDRMEG-----ARFRHTAQFNRWRPDRDPRSCSYAQLERPL 346
LigC2           AGKDLSFVPLRPERLVEVRYDHMEG-----RRFRHTAQFNRWRPDRDARSCTFAQLDSPP 344
                 . *  :.*****:: ** **:***      ****:.:* ******:. **:: **: *

LigC1           NYDLYDVLEEQ- 348
LigCTB          TVSLSDIVPGLR 358
LigC2           HSSSVTSCRV-- 354
                  .
```

Figure S5

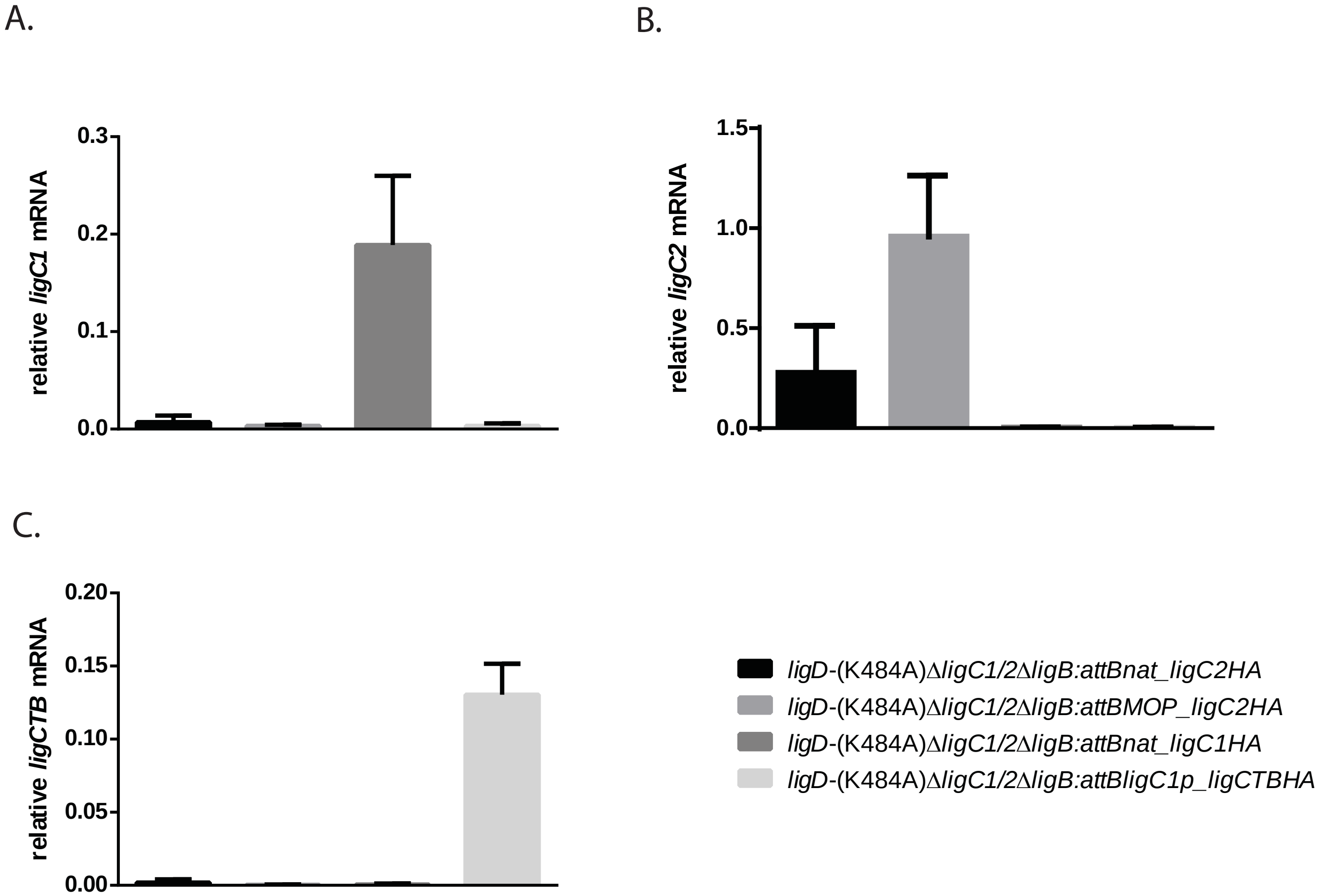
A.
relative *ligC1* mRNA
0.3
0.2
0.1
0.0
B.
relative *ligC2* mRNA
1.5
1.0
0.5
0.0
C.
relative *ligCTB* mRNA
0.20
0.15
0.10
0.05
0.00
ligD-(K484A)Δ*ligC1/2*Δ*ligB:attBnat_ligC2HA*
ligD-(K484A)Δ*ligC1/2*Δ*ligB:attBMOP_ligC2HA*
ligD-(K484A)Δ*ligC1/2*Δ*ligB:attBnat_ligC1HA*
ligD-(K484A)Δ*ligC1/2*Δ*ligB:attBligC1p_ligCTBHA*

Figure S6

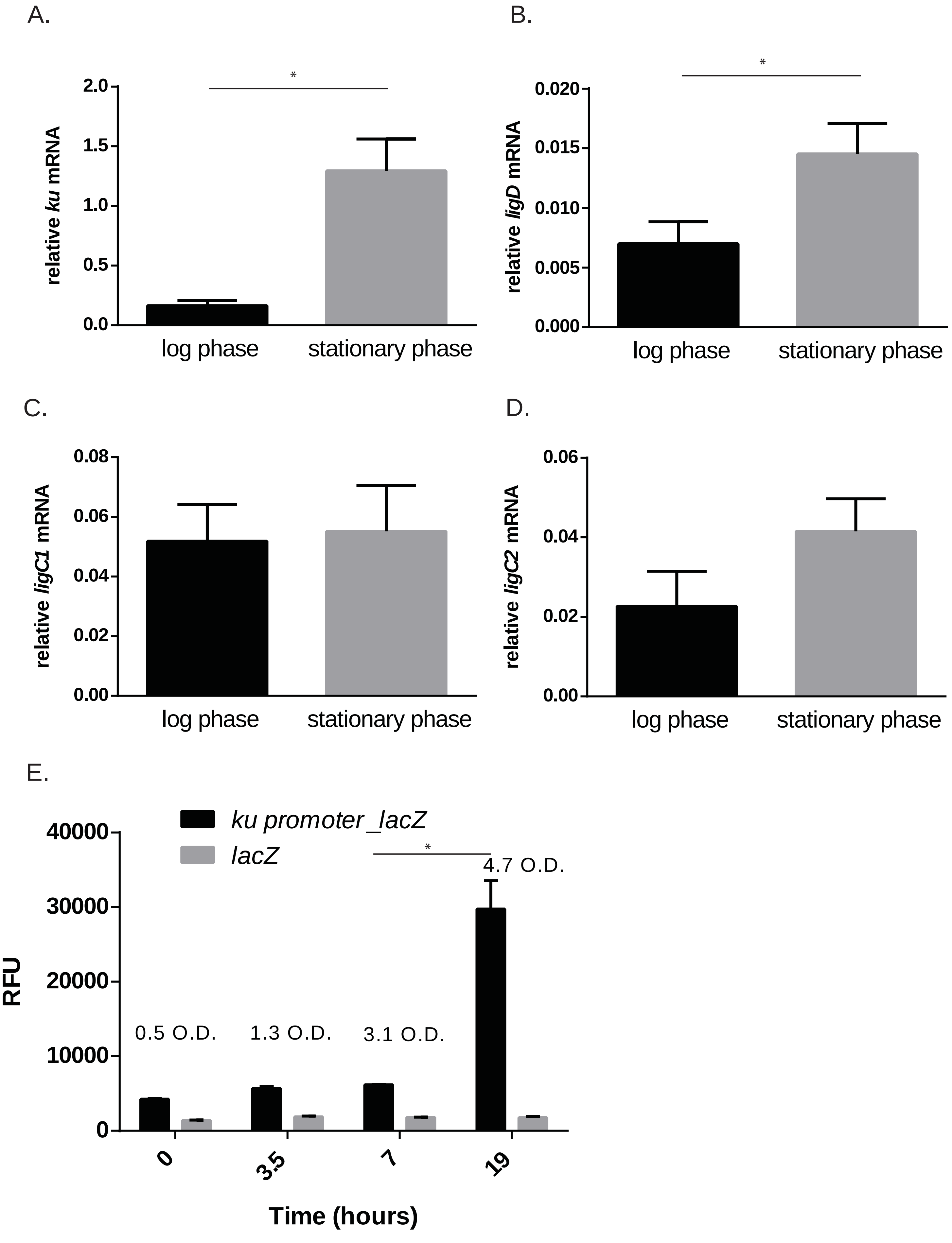
A.
relative ku mRNA
2.0
1.5
1.0
0.5
0.0
*
log phase
stationary phase
B.
relative ligD mRNA
0.020
0.015
0.010
0.005
0.000
*
log phase
stationary phase
C.
relative ligC1 mRNA
0.08
0.06
0.04
0.02
0.00
log phase
stationary phase
D.
relative ligC2 mRNA
0.06
0.04
0.02
0.00
log phase
stationary phase
E.
ku promoter_lacZ
lacZ
RFU
40000
30000
20000
10000
0
*
0.5 O.D.
1.3 O.D.
3.1 O.D.
4.7 O.D.
0
3.5
7
19
Time (hours)

Figure S7