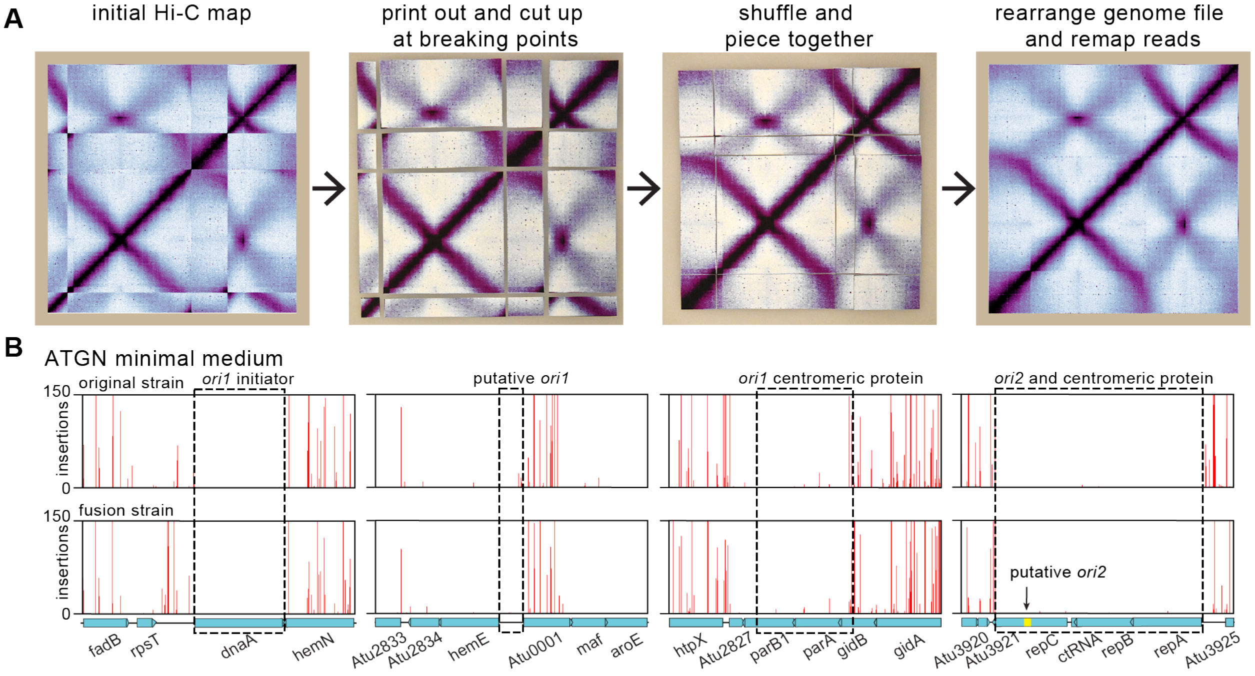


Figure S1. ***A. tumefaciens*** **fusion strain has a dicentric linear chromosome. Related to Figure 1 and Figure 2.**

(A) Deciphering the genome rearrangement. The Hi-C map generated by mapping Hi-C reads from *A. tumefaciens* C58 fusion strain to the original C58 reference genome (first panel) was printed out, cut up at breakpoints (second panel), and reassembled to generate a confluent picture (third panel). The pattern indicated that the circular Ch1 and linear Ch2 were fused into a large linear chromosome. When the same Hi-C data were mapped to the rearranged reference sequence, the same pattern was seen (fourth panel). **(B)** The initiator proteins, replication origin sequences, and partitioning systems of both *ori1* and *ori2* are required for the survival of the original and fusion strains. Tn-seq plots showing transposon intolerance at indicated regions in the original strain (top) and the fusion strain (bottom). This screen was carried out in minimal ATGN medium.

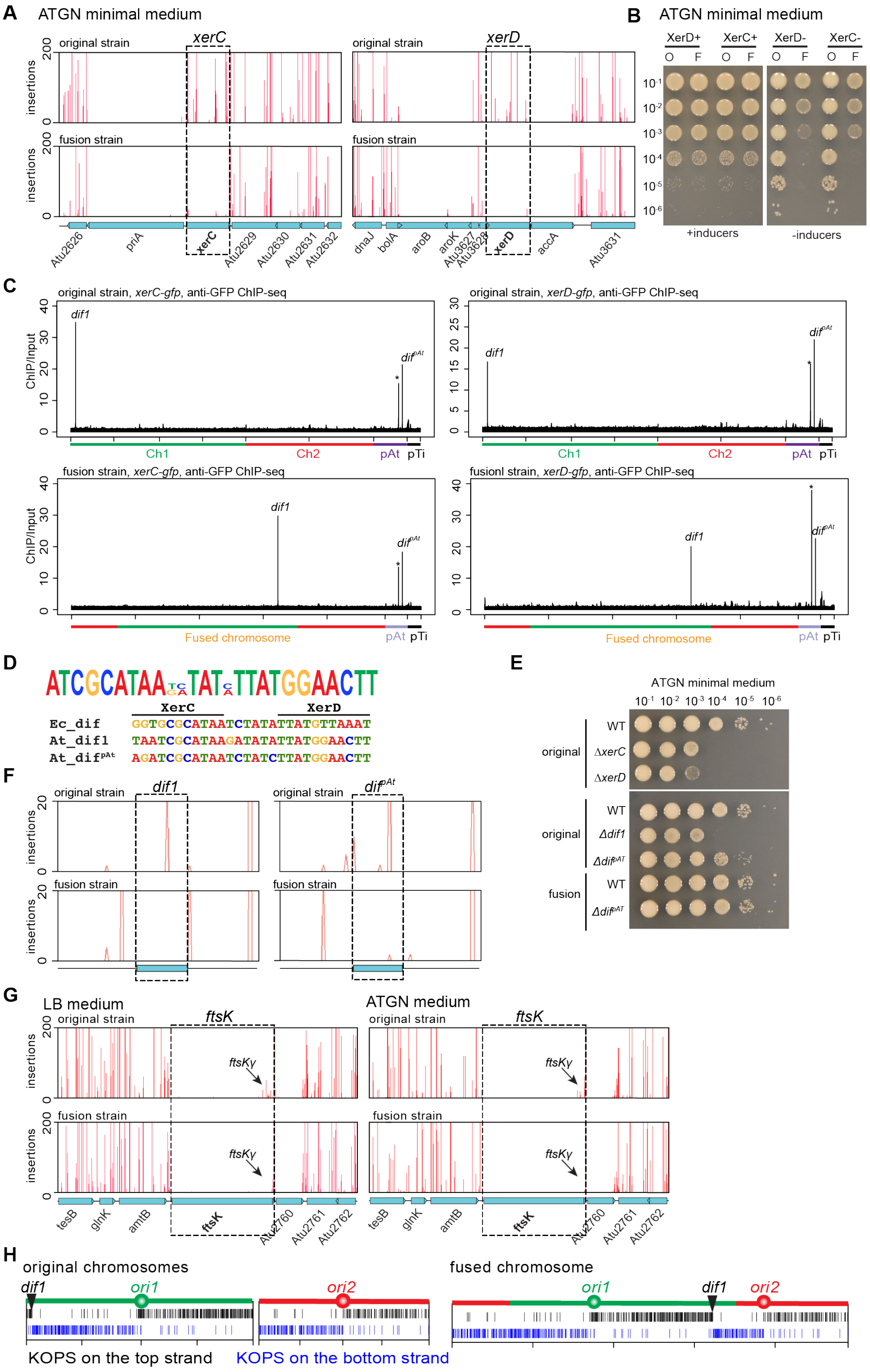

A
ATGN minimal medium
original strain
xerC
fusion strain
insertions
200
0
Atu2626
priA
xerC
Atu2629
Atu2630
Atu2631
Atu2632
original strain
xerD
fusion strain
dnaJ
bolA
aroB
aroK
Atu3627
Atu3628
xerD
accA
Atu3631
B
ATGN minimal medium
XerD+
XerC+
XerD-
XerC-
O
F
10-1
10-2
10-3
10-4
10-5
10-6
+inducers
-inducers
C
original strain, xerC-gfp, anti-GFP ChIP-seq
ChIP/Input
dif1
difpAt
*
Ch1
Ch2
pAt
pTi
original strain, xerD-gfp, anti-GFP ChIP-seq
fusion strain, xerC-gfp, anti-GFP ChIP-seq
fusionl strain, xerD-gfp, anti-GFP ChIP-seq
Fused chromosome
D
ATCGCATAATATTATGGAACTT
XerC
XerD
Ec_dif GGTGCGCATAATCTATATTATGTTAAAT
At_dif1 TAATCGCATAAGATATATTATGGAACTT
At_difpAt AGATCGCATAATCTATCTTATGGAACTT
E
ATGN minimal medium
10-1 10-2 10-3 10-4 10-5 10-6
original
WT
ΔxerC
ΔxerD
original
WT
Δdif1
ΔdifpAt
fusion
WT
ΔdifpAT
F
original strain
dif1
difpAt
fusion strain
insertions
20
0
G
LB medium
ATGN medium
original strain
fusion strain
ftsK
ftsKγ
tesB
glnK
amtB
ftsK
Atu2760
Atu2761
Atu2762
H
original chromosomes
fused chromosome
dif1
ori1
ori2
KOPS on the top strand
KOPS on the bottom strand

Figure S2. Binding sites of XerC and XerD in the original and fusion strains. Related to Figure 3.

(A) Tn-seq plots showing transposon insertions at *xerC/xerD* loci in the WT original strain (top) and the WT fusion strain (bottom) growing on ATGN plates.

(B) Validation of essentiality of *xerD* and *xerC* using depletion constructs, from left to right: AtWX332, AtWX327, AtWX331 and AtWX323. 10-fold serial dilutions of indicated strains were spotted on ATGN plates. The depletion constructs had the endogenous promotor of *xerD* or *xerC* replaced by *Plac-riboswitch*. 2 mM theophylline and 0.5 mM IPTG were added as inducers.

(C) ChIP enrichment of XerC-GFP and XerD-GFP in the original and fusion strains: top left, AtWX377 (original *xerC-gfpmut3*); top right, AtWX370 (original *xerD-gfpmut3*); bottom left, AtWX367 (fusion *xerC-gfpmut3*); bottom right, AtWX379 (fusion *xerD-gfpmut3)*. The original strains were mapped to the original reference genome with *ori1* in the middle of Ch1, and the fusion strains were mapped to the re-arranged reference genome. x-axis shows genome positions and y-axis indicates ChIP enrichment (ChIP/input) in 1-kb bins. Asterisks indicate a systematic peak we observed for every anti-GFP ChIP-seq experiment.

(D) Top, logo of XerC/XerD binding sequence extracted from ChIP-seq peaks in (**C**). Bottom, comparison of *A. tumefaciens dif1* and *dif*^{pAt} with *E. coli dif* with indicated XerC and XerD binding arms.

(E) 10-fold serial dilutions of the indicated strains spotted on ATGN plates, from top to bottom: AtWX063, AtWX375, AtWX092, AtWX063, AtWX439, AtWX441, AtWX001 and AtWX440.

(F) Transposon insertions at *dif1* (left) and *dif*^{pAt} (right) in the WT original strain (top) and the WT fusion strain (bottom) growing on LB plates.

(G) Tn-seq plots showing transposon insertions at *ftsK* locus in the WT original strain (top) and the WT fusion strain (bottom) growing on LB plates (left) and ATGN plates (right).

(H) Distributions of FtsK orienting polar sequences (KOPS) in original chromosomes (left) and the fused chromosome (right). KOPS (GGGNAGGG) on the top (Watson) strand and on the bottom (Crick) strand are shown as black and blue bars, respectively.

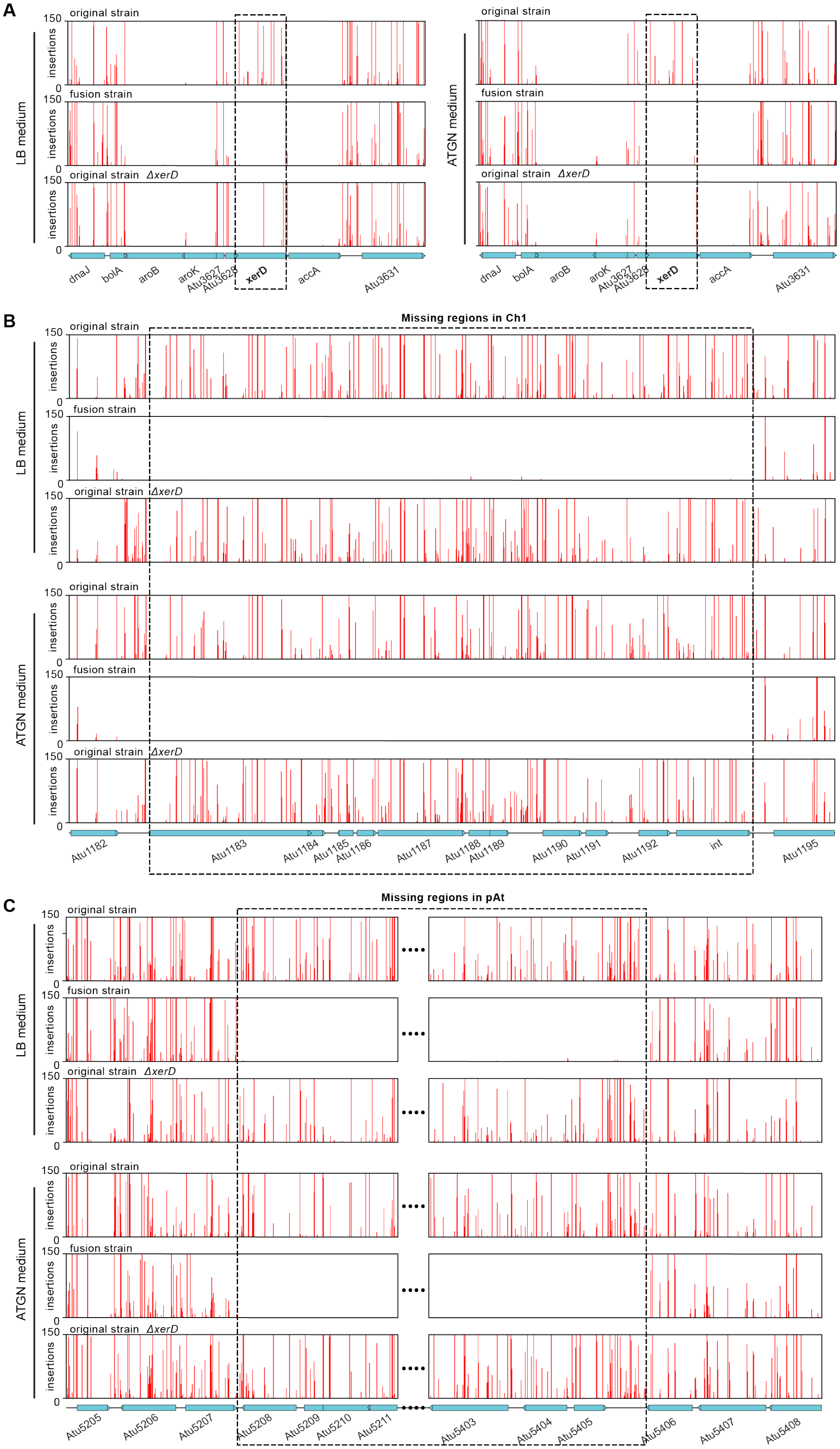

A
original strain
fusion strain
original strain ΔxerD
LB medium
ATGN medium
insertions
150
0
dnaJ
bolA
aroB
aroK
Atu3627
Atu3628
xerD
accA
Atu3631
B
Missing regions in Ch1
Atu1182
Atu1183
Atu1184
Atu1185
Atu1186
Atu1187
Atu1188
Atu1189
Atu1190
Atu1191
Atu1192
int
Atu1195
C
Missing regions in pAt
Atu5205
Atu5206
Atu5207
Atu5208
Atu5209
Atu5210
Atu5211
Atu5403
Atu5404
Atu5405
Atu5406
Atu5407
Atu5408

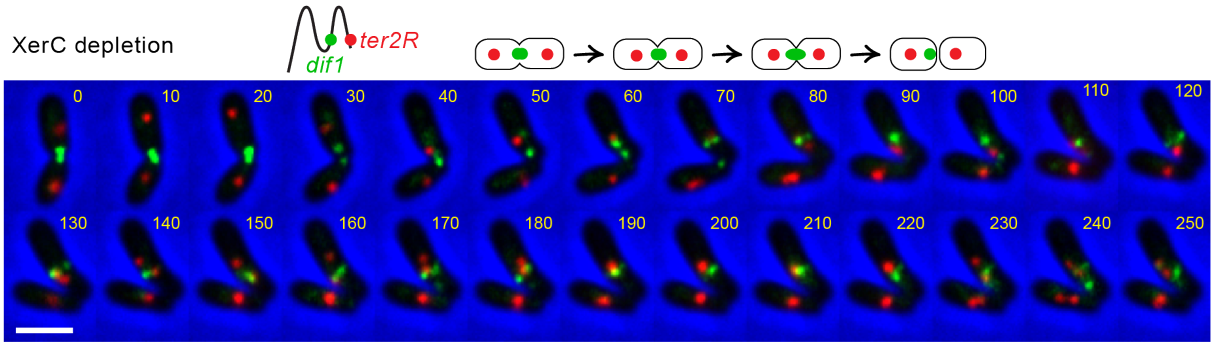
Figure S3. *xerD* is not synthetically lethal with genes that are missing on Ch1 and pAt in the fusion strain. Related to Figure 3. Tn-seq was performed in three different strains: original strain (AtWX063, top), fusion strain (AtWX001, middle), and original ∆*xerD* strain (AtWX092, bottom). Cells were growing on LB or ATGN plates as indicated.

(A) Transposon insertions at *xerD* locus. *xerD* was successfully deleted from the original strain as indicated by no insertions at this locus in original ∆*xerD* (bottom).

(B) Transposon insertions at the Ch1 region that is missing on the fused chromosome. ∆*xerD* cells tolerated insertions at these regions (bottom), suggesting *xerD* is not synthetically lethal with these missing genes.

(C) Transposon insertions at the pAt region that is missing from the fusion strain. ∆*xerD* cells tolerated insertions at these regions (bottom), suggesting *xerD* is not synthetically lethal with these missing genes. Only the junctions are shown.

A



B

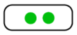
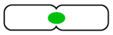
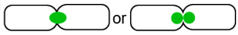
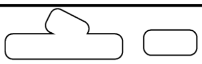
cell types \ depletion time / No. of cells analyzed	WT	XerC depletion				XerD depletion			
	0h 1154 cells	0h 902 cells	3h 1163 cells	6h 1004 cells	12h 828 cells	0h 905 cells	3h 942 cells	6h 980 cells	12h 715 cells
growing cell with one focus 	67.7%	64.5%	65.0%	44.7%	24.0%	53.7%	57.1%	49.0%	20.3%
growing cell with two foci 	4.8%	6.3%	3.6%	4.5%	2.1%	10.5%	7.0%	7.4%	3.5%
early dividing cell 	8.5%	10.9%	11.5%	11.1%	7.0%	14.4%	10.9%	14.4%	4.6%
deeply constricted cell with one or two foci 	12.0%	8.6%	8.1%	22.7%	21.5%	10.4%	13.4%	20.7%	18.3%
cells without foci 	5.9%	7.9%	10.1%	11.9%	38.2%	7.1%	8.9%	6.4%	49.5%
cell with foci and abonornal cell shape 	1.2%	1.8%	1.7%	5.2%	7.2%	4.0%	2.7%	2.0%	3.8%

Figure S4. Quantitative analysis of *dif1* localization in XerC/D depletion strains. Related to Figure 4.

(A) Time-lapse progression of XerC depletion strain containing *dif1* (green) and *ter2R* (red) on the fused chromosome (AtWX514). *dif1* was visualized as shown in **Figure 4B** whereas *ter2R* was visualized by inserting visualization cassette, *mcherry-parBP1-parSP1*, at 39.8 kb away from *ter2R.*

(B) Quantitative analysis of *dif1* localization patterns in the WT fusion strain (AtWX455), XerC-depleted (AtWX467) and XerD-depleted (AtWX471) fusion strains. Snapshot images of cells growing in LB medium were collected at 0 h, 3 hr, 6 hr, 12 hr upon the depletion. Cells were classified and quantified according to cell cycle and the number of foci. After 6 hr depletion, the percentage of deeply constricted cells increased by about two fold. Upon 12 hr depletion, the proportion of cells without foci rose by over three fold.

Lab	Isolate name	Configuration	References
L.M. Banta	C58	fusion	Banta Lab collection
P. Brown	C58	fusion	[S1]
C. Fuqua	C58	fusion	[S2]
S.K. Farrand	C58	fusion	[S3]
X. Wang	C58 fusion (AtWX001)	fusion	This study
S.C. Winans	C58	fusion	[S4]
P. Zambryski	C58	fusion	[S5]
J.H. Chang	C58 A8	original	Chang Lab collection
S.K. Farrand	C58 C1RS, C58 UIA5, C58 NTL4	original	[S6 S7]
C. Fuqua	C58	original	[S8]
S.B. Gelvin	C58	original	Gelvin Lab collection
B. Goodner	C58 BG	original	[S9]
R. Hallez	C58	original	Hallez Lab collection
C.I. Kado	C58 NT1REB	original	[S10]
E-M. Lai	C58 A107	original	[S11]
K.S. Mysorel	C58	original	[S12]
E.W. Nester	C58 A136, C58-3, EN58	original	[S13 S14]
X. Wang	C58 original (AtWX063)	original	[S15]
P. Zambryski	C58	original	[S16]

Table S1. Chromosomal configuration of *A. tumefaciens* strains in different labs. Related to STAR Methods.

Sample name	Figure	Reference	Identifier
401_Wang_HiC_AtWX063_ATGN	1A	S15	GSM5542437
438_Wang_WGS_AtWX063_ATGN_rep2	1A	S15	GSM5689555
500_Wang_HiC_AtWX001_ATGN	1BC, S1A	This study	SAMN27387414
501_Wang_WGS_AtWX001_ATGN	1BC	This study	SAMN27387415
502_Wang_Tnseq_AtWX063_LB	2D, 3A, S2F, S3	This study	SAMN27387416
503_Wang_Tnseq_AtWX001_LB	2D, 3A, S2F, S3	This study	SAMN27387417
504_Wang_Tnseq_AtWX387_LB	3C	This study	SAMN27387418
505_Wang_Tnseq_AtWX398_LB	3C	This study	SAMN27387419
506_Wang_Tnseq_AtWX035_LB	3D	This study	SAMN27387420
507_Wang_Tnseq_AtWX108_LB	3D	This study	SAMN27387421
508_Wang_Tnseq_AtWX063_ATGN	S1B, S2A, S3	This study	SAMN27387422
509_Wang_Tnseq_AtWX001_ATGN	S1B, S2A, S3	This study	SAMN27387423
510_Wang_ChIP_antiGFP_AtWX367_ATGN	S2C	This study	SAMN27387424
511_Wang_input_AtWX367_ATGN	S2C	This study	SAMN27387425
512_Wang_ChIP_antiGFP_AtWX370_ATGN	S2C	This study	SAMN27387426
513_Wang_input_AtWX370_ATGN	S2C	This study	SAMN27387427
514_Wang_ChIP_antiGFP_AtWX377_ATGN	S2C	This study	SAMN27387428
515_Wang_input_AtWX377_ATGN	S2C	This study	SAMN27387429
516_Wang_ChIP_antiGFP_AtWX379_ATGN	S2C	This study	SAMN27387430
517_Wang_input_AtWX379_ATGN	S2C	This study	SAMN27387431
518_Wang_Tnseq_AtWX092_LB	S3	This study	SAMN27387432
519_Wang_Tnseq_AtWX092_ATGN	S3	This study	SAMN27387433

Table S2. Next generation sequencing samples used in this study. Related to STAR Methods.

Strains used in main figures			
Strain	**Genotype**	**Reference**	**Figure**
AtWX001	C58 fusion, wild type	This study	1BCEF, 2BCD, 3A, S1AB, S2AEFGH, S3,
AtWX063	C58 original, wild type	[S15]	1ADF, 2BCD, 3A, S1B, S2AEFGH, S3
AtWX263	C58 original, carrying pWX970, pSRKKm *Plac rfp-repB*^{Ch2} (Atu3923/ATU_RS18280) *terminator Plac egfp-parB1* (Atu2828/ATU_RS13770) *terminators*	[S15]	2A
AtWX366	C58 fusion, carrying pWX970, pSRKKm *Plac rfp-repB*^{Ch2} (Atu3923/ATU_RS18280) *terminator Plac egfp-parB1* (Atu2828/ATU_RS13770) *terminators*	This study	2A
AtWX025	C58 fusion, *tetRA::a-attTn7 pLac repB*^{ch2} *gen*, Δ*repB*^{Ch2}	This study	2C
AtWX089	C58 original, Δ*repB*^{Ch2} (Atu3923/ATU_RS18280)::amp	[S15]	2C
AtWX192	C58 original, *tetRA::gen PtraI-riboswitch-parB1*(Atu2828/ATU_RS13770) *traR*	[S15]	2C
AtWX194	C58 fusion, *tetRA::gen PtraI-riboswitch-parB1*(Atu2828/ATU_RS13770) *traR*	This study	2C
AtWX323	C58 fusion, *xerC* (Atu2628/ATU_RS12790)*::terminator gen LacI terminators Plac riboswitch xerC*	This study	3B, S2B
AtWX327	C58 fusion, *xerD* (Atu3629/ATU_RS16850)*::terminator gen lacI terminators Plac riboswitch xerD*	This study	3B, S2B
AtWX331	C58 original, *xerC* (Atu2628/ATU_RS12790)*::terminator gen LacI terminators Plac riboswitch xerC*	This study	3B, S2B
AtWX332	C58 original, *xerD* (Atu3629/ATU_RS16850)*::terminator gen lacI terminators Plac riboswitch xerD*	This study	3B, S2B
AtWX387	C58 fusion, Δ*recA* (Atu1873/ATU_RS09160)::*amp*	This study	3C
AtWX398	C58 original, Δ*recA* (Atu1873/ATU_RS09160)::*amp*	This study	3C
AtWX035	C58 fusion, Δ*smc* (Atu0801/ATU_RS03945)	This study	3D
AtWX108	C58 original, Δ*smc* (Atu0801/ATU_RS03945)	[S15]	3D
AtWX502	C58 fusion, *xerC* (Atu2628/ATU_RS12790)*::terminator gen LacI* terminators *Plac riboswitch xerC,* carrying pWX1076, pSRKKm *cymR cuO Plac cuO At parC* (Atu1158/ATU_RS05720) *At parE* (Atu1622/ATU_RS07965)	This study	3E
AtWX503	C58 fusion, *xerD* (Atu3629/ATU_RS16850)*::terminator gen lacI terminators Plac riboswitch xerD,* carrying pWX1076, pSRKKm *cymR cuO Plac cuO At parC* (Atu1158/ATU_RS05720) *At parE* (Atu1622/ATU_RS07965)	This study	3E
AtWX540	C58 fusion, *xerC* (Atu2628/ATU_RS12790)*::terminator gen LacI*	This study	3E

	terminators *Plac riboswitch xerC,* carrying pWX1080, pSRKKm *cymR cuO Plac cuO Ec parE-parC*		
AtWX541	C58 fusion, *xerD* (Atu3629/ATU_RS16850)*::terminator gen lacI terminators Plac riboswitch xerD,* carrying pWX1080, pSRKKm *cymR cuO Plac cuO Ec parE-parC*	This study	3E
AtWX455	C58 fusion, *ygfp-parB*^{MT1}*-parS*^{MT1} inserted between Atu1460/ATU_RS07195 and Atu1461/ ATU_RS07200, 57 kb from *dif1*	This study	4B, S4B
AtWX467	C58 fusion, *xerC* (Atu2628/ATU_RS12790)*::terminator gen LacI terminators Plac riboswitch xerC*; *ygfp-parB*^{MT1}*-parS*^{MT1} inserted between Atu1460/ATU_RS07195 and Atu1461/ ATU_RS07200, 57 kb from *dif1*	This study	4CD, S4B
AtWX471	C58 fusion, *xerD* (Atu3629/ATU_RS16850)*::terminator gen lacI terminators Plac riboswitch xerD; ygfp-parB*^{MT1}*-parS*^{MT1} inserted between Atu1460/ATU_RS07195 and Atu1461/ ATU_RS07200, 57 kb from *dif1*	This study	4D, S4B
Strains used for strain building or in supplemental figures			
Strain	**Genotype**	**Reference**	**Figure**
AtWX367	C58 fusion*, xerC-gfpmut3* (Atu2628/ATU_RS12790)	This study	S2C
AtWX370	C58 original, *xerD-gfpmut3* (Atu3629/ATU_RS16850)	This study	S2C
AtWX377	C58 original*, xerC-gfpmut3* (Atu2628/ATU_RS12790)	This study	S2C
AtWX379	C58 fusion, *xerD-gfpmut3* (Atu3629/ATU_RS16850)	This study	S2C
AtWX439	C58 original, Δ*dif1*::*amp*	This study	S2E
AtWX440	C58 original, Δ*dif*^{pAt}::*amp*	This study	S2E
AtWX441	C58 fusion, Δ*dif1*::*amp*	This study	S2E
AtWX092	C58 original, Δ*xerD* (Atu3629/ATU_RS16850)::*amp*	This study	S2E, S3
AtWX375	C58 original, Δ*xerC* (Atu2628/ATU_RS12790)::*amp*	This study	S2E
AtWX514	C58 fusion, *xerC* (Atu2628/ATU_RS12790)*::terminator gen LacI terminators Plac riboswitch xerC*; *ygfp-parB*^{MT1}*-parS*^{MT1} inserted between Atu1460/ATU_RS07195 and Atu1461 57 kb from *dif1; mCherry-parB*^{P1}*-parS*^{P1} inserted between Atu4854 and Atu4856 39.8 kb from *ter2R*	This study	S4A
Plasmids used in this study			
Plasmid	**Description**	**Reference**	
pWX811	pNPTS138 Δ *repB*^{Ch2} (Atu3923/ATU_RS18280)::*amp*	This study	
pWX813	pMiniTn7 pLac *At repB*^{Ch2} (Atu3923/ATU_RS18280)	This study	
pWX832	pNPTS138 Δ*smc* (Atu0801/ATU_RS03945)	[S15]	
pWX854	pNPTS138 Δ*repB*^{Ch2} (Atu3923/ATU_RS18280)::*amp*	[S15]	
pWX855	pNPTS138 Δ*xerD* (Atu3629/ATU_RS16850)::*amp*	This study	
pWX885	pMiniTn7 *PtraI-riboswitch-parB1*(Atu2828/ATU_RS13770) *traR* (Atu6134: ATU_RS23655)	[S15]	

pWX902	pNPTS138 *tetRA::gen PtraI-riboswitch-parB1*(Atu2828/ATU_RS13770) *traR*	[S15]	
pWX914	pNPTS138 Δ*parB1*(Atu2828/ATU_RS13770)::*amp*	[S15]	
pWX916	pACYC *terminator Ppen cfp-parB*^{P1}*-parS*^{P1} (*amp*)	[S17]	
pWX923	pACYC *terminator Ppen CFP-ParBP1 parSP1 terminators*	This study	
pWX926	pNPTS138 *Ppen ygfp-parB*^{MT1}*-parS*^{MT1} at Atu1460/ATU_RS07195	This study	
pWX930	pNPTS138 *Ppen cfp-parB*^{P1}*-parS*^{P1} at Atu3054/ATU_RS14060	[S17]	
pWX936	pNPTS138 *PT7strong cfp-parB*^{P1}*-parS*^{P1} at Atu3054/ATU_RS14060	[S17]	
pWX943	pNPTS138 *terminator gen lacI terminators Plac-riboswitch*	This study	
pWX944	pNPTS138 *terminator gen LacI terminators Plac riboswitch xerC* (Atu2628/ATU_RS12790)	This study	
pWX945	pNPTS138 *terminator gen LacI terminators Plac riboswitch xerD* (Atu3629/ATU_RS16850)	This study	
pWX965	pNPTS138 *PT7strong ygfp-parB*^{MT1}*-parS*^{MT1} at Atu1460/ATU_RS07195	This study	
pWX968	pNPTS138 *PT7strong cfp-parB*^{P1}*-parS*^{P1} at Atu4854	This study	
pWX970	pSRKKm *Plac rfp-repB*^{Ch2} (Atu3923/ATU_RS18280) *terminator Plac egfp-parB1* (Atu2828/ATU_RS13770) *terminators*	[S15]	
pWX998	pNPTS138 *PT7strong mcherry-parB*^{P1}*-parS*^{P1} at Atu4854	This study	
pWX1006	pNPTS138 Δ*xerC* (Atu2628/ATU_RS12790)::*amp*	This study	
pWX1007	pNPTS138 Δ*recA* (Atu1873/ATU_RS09160)::*amp*	This study	
pWX1008	pNPTS138 *xerC-gfpmut3* (Atu2628/ATU_RS12790)	This study	
pWX1009	pNPTS138 *xerD-gfpmut3* (Atu3629/ATU_RS16850)	This study	
pWX1039	pNPTS138 Δ*dif1*::*amp*	This study	
pWX1040	pNPTS138 Δ*dif*^{pAt}::*amp*	This study	
pWX1076	pSRKKm *cymR cuO Plac cuO At parC* (Atu1158/ATU_RS05720) *At parE* (Atu1622/ATU_RS07965)	This study	
pWX1080	pSRKKm *cymR cuO Plac cuO Ec parE-parC*	This study	
pHP45omega	*omega-amp cassette*	[S18]	
pNPTS138	*oriT sacB kan*	[S19]	
pSRKKm	Broad host-range, *Plac*	[S20]	
cumate induction system	pSRKKm *cymR cuO Plac cuO msfgfp*	[S21] gift from Pam Brown	
pLEXRparEC3	*E. coli parE parC* expressed from IPTG inducible promotor	[S22]	

Table S3. Bacterial strains and plasmids used in this study. Related to STAR Methods.

Oligos	**Sequence**	**Use**
oWX0487	aacggtctgataagagacaccggc	sequencing
oWX1854	cgccagggttttcccagtcacgac	sequencing
oWX1855	tcacacaggaaacagctatgac	sequencing
oWX2021	tggcgccaagcttctctgcaggatcaggcgcagaacttctacgatgtc	pWX811
oWX2022	ggaccggtattcggcaaacagcgtaagattggcgaatatctgttttc	pWX811
oWX2023	gaaaacagatattcgccaatcttacgctgtttgccgaataccggtcc	pWX811
oWX2024	gctagcgaattcgtggatccagatcgcgcagagacaattgcgtgtttg	pWX811
oWX2027	gcgcatatgagcagaaaacagatattcgcc	pWX813
oWX2028	tttctcgagtcactgcttggaccggtattcggc	pWX813
oWX2029	acaaatccgccgctaggagcttgc	sequencing
oWX2031	gaactgggtgtagcgtcgtaagc	sequencing
oWX2042	gcgatccggtatgaaggatggatc	sequencing
oWX2144	tggcgccaagcttctctgcaggattagtgtcgttgataaatgcg	pWX855
oWX2146	cccatgagcggacaaccgatggcAggcaaaaaccttgattagagcgac	pWX855
oWX2148	ctgcaaatgtttacgaagcg	Sequencing
oWX2149	gaatagatatcctgcatcgc	Sequencing
oWX2208	aaataaacaaataggggttccgcgtgccatcggttgtccgctca	pWX855
oWX2209	gcctcactgattaagcattggtaaggcaaaaaccttgattagag	pWX855
oWX2210	cgcggaacccctatttgttta	pWX1006/pWX1007
oWX2211	ttaccaatgcttaatcagtgaggc	pWX1006/PWX1007
oWX2377	ggcttcctttgttatcaagcgcag	sequencing
oWX2379	ctttttccatcagctctgttaccg	sequencing
oWX2395	tcttcgctattacgccagatcc	sequencing
oWX2397	gatgacggtaactacaaaaccc	sequencing
oWX2403	catggatccatgtcgacagactctctagcttgaggcatc	pWX923
oWX2404	gctcagccggccgtcaacgttcttgccattgctgca	pWX923
oWX2407	ctctagatagcgcatgctgaattc	pWX926/pWX968
oWX2408	ggttatgctagttattgctcagcc	pWX926/pWX968
oWX2409	ggcgccaagcttctctgcaggatatcgaaatatgctccgaaaaatatgc	pWX926
oWX2410	gaattcagcatgcgctatctagagtcagcgcaccagccggatgtcgc	pWX926
oWX2411	ggctgagcaataactagcataacccaagcggccccagtgccgcgtcc	pWX926
oWX2412	ctagcgaattcgtggatccagattggttgagctatcgcgatcatggc	pWX926
oWX2418	atatcgcggaggatggcgacc	sequencing
oWX2419	ccttctcgtggtcaagcgtgc	sequencing
oWX2424	ttctgccgctccgatcaaaacagg	sequencing
oWX2425	ccggactccacatccgcagatttc	sequencing
oWX2426	ggccttctgcttagctagagcggc	sequencing
oWX2426	ggccttctgcttagctagagcggc	sequencing
oWX2431	taatacgactcactatagggagaccacaacgcttcatctggttacgatcaatc	pWX965
oWX2432	ggtctccctatagtgagtcgtattaatttcgaaatgatgacctcgtttccacc	pWX965
oWX2450	gtacctatagtgagtcgtatgaaattgttatccgctcaca	pWX943
oWX2451	tgtgagcggataacaatttcatacgactcactataggtac	pWX943
oWX2452	gctagcgaattcgtggatccagatcttgttgatacccccctgcgc	pWX943
oWX2453	tggcgccaagcttctctgcaggataagaccgtctcgaacacgcc	pWX944
oWX2456	tcggtcacgcggtactccatcttgttgatacccccctgcgc	pWX944
oWX2457	gcgcaggggggtatcaacaagatggagtaccgcgtgaccga	pWX944
oWX2458	gctagcgaattcgtggatccagatcaggaacatcggttcgtccg	pWX944
oWX2459	tggcgccaagcttctctgcaggatttcagccacagcgaaatgcc	pWX945
oWX2462	gccatcggttgtccgctcatcttgttgatacccccctgcgc	pWX945

oWX2463	gcgcagggggtatcaacaagatgagcggacaaccgatggc	pWX945
oWX2464	gctagcgaattcgtggatccagataccttccttactgttcgagg	pWX945
oWX2482	agcttctctgcaggataaaaaaaaaaccccgctttcgcggggttaggtggcggtacttggg	pWX943
oWX2483	cgcgaaagcggggttttttttttccatcccccgaaactatgc	pWX944
oWX2484	gcatagtttcgggggatggaaaaaaaaaaccccgctttcgcg	pWX944
oWX2485	cgaaagcggggttttttttttcatggggtgcgccttgccg	pWX945
oWX2486	cggcaaggcgcaccccatgaaaaaaaaaaccccgctttcgcg	pWX945
oWX2497	aagtcaagtttgaaggtgataccc	sequencing
oWX2491	catcaaacatcgacccacgg	sequencing
oWX2492	gaacgatgccctcattcagc	sequencing
oWX2493	tgcgacatcgtataacgttactgg	sequencing
oWX2514	cgccaagcttctctgcaggatatctcgccaattagaatggcctgtg	pWX968
oWX2515	aattcagcatgcgctatctagagttaaatagtggtcgtcctcgaaag	pWX968
oWX2516	ctgagcaataactagcataacccgctgctgtcgtcggcgcctactatc	pWX968
oWX2517	agcgaattcgtggatccagatatccgtcatcgatcagcaattcgatac	pWX968
oWX2518	tgtcaccacctgccagaacaagct	sequencing
oWX2519	ggcaagttcatcaaagacctgcga	sequencing
oWX2584	ttcaagcttaaaggaggtggaaacatggtcagcaagggagaggaag	pWX998
oWX2585	ctgctcgaccatgagctcgaattctttgtataattcgtccattccacc	pWX998
oWX2589	cttcctctcccttgctgaccatgtttccacctcctttaagcttg	pWX998
oWX2590	ggtggaatggacgaattatacaaagaattcgagctcatggtcgagcag	pWX998
oWX2601	cgccaagcttctctgcaggatatccagaaagaccgtctcgaacacgcc	pWX1006
oWX2602	aaataaacaaataggggttccgcgcacgcggtactccattccatcccc	pWX1006
oWX2603	gcctcactgattaagcattggtaagaaatctacgacaacgcccaccc	pWX1006
oWX2604	ctagcgaattcgtggatccagatatccagcatccagggcttcatgcgcg	pWX1006
oWX2605	ggcgccaagcttctctgcaggatatcgatgatgcggtcatcggaaaaac	pWX1007
oWX2606	aaataaacaaataggggttccgcgggcagcgacgagggctgatagattc	pWX1007
oWX2607	gcctcactgattaagcattggtaaagaattttgtgccatccgaaccac	pWX1007
oWX2608	gctagcgaattcgtggatccagatatccgtcagcgtatcctcgggcag	pWX1007
oWX2609	tggcgccaagcttctctgcaggatatctgcgtgcttttctggccaac	pWX1008
oWX2610	tcctttactggatcctccagatccggcacgtgggtgggcgttgtcg	pWX1008
oWX2611	ggatctggaggatccagtaaaggag	pWX1008/pWX1009
oWX2612	ttatttgtatagttcatccatgcc	pWX1008/pWX1009
oWX2613	ggcatggatgaactatacaaataaagcaggaattggttaaccgtaaac	pWX1008
oWX2614	gctagcgaattcgtggatccagatagaaaggagagaccggttgccttg	pWX1008
oWX2615	tggcgccaagcttctctgcaggatctttctttattcggaagggctgc	pWX1009
oWX2616	tcctttactggatcctccagatccatcaaggtttttgccctgtttggc	pWX1009
oWX2617	ggcatggatgaactatacaaataaagcgaccggaaccgccggataatc	pWX1009
oWX2618	gctagcgaattcgtggatccagatcgaatgttcgagcatataaacgcg	pWX1009
oWX2624	ggataataccgcgccacatagcag	sequencing
oWX2625	tgcgcaaactattaactggcgaac	sequencing
oWX2626	gtaatacgactcactagtggggcc	sequencing
oWX2627	gataatggtctgctagttgaacgc	sequencing
oWX2628	ttgactagagggtcgacgcatgcc	sequencing
oWX2720	tgaccgcttcctcgtgctttacgg	sequencing
oWX2796	tggcgccaagcttctctgcaggattcgggttcttgataagaacggtg	pWX1039
oWX2797	aaataaacaaataggggttccgcgttgtttgtagctgggtacattc	pWX1039
oWX2798	tagaatgtacccagctacaaacaacgcggaacccctatttgtttattt	pWX1039
oWX2799	aaatcctaggtttcagagacaaatttaccaatgcttaatcagtgaggc	pWX1039

oWX2800	gcctcactgattaagcattggtaaatttgtctctgaaacctaggatttg	pWX1039
oWX2801	gctagcgaattcgtggatccagatctctgcgcccggcattttcgacc	pWX1039
oWX2802	tggcgccaagcttctctgcaggattcgggttcttgataagaacggtg	pWX1040
oWX2803	aaataaacaaataggggttccgcgaaatagggaattgggtacattc	pWX1040
oWX2804	tagaatgtacccaattccctatttcgcggaacccctatttgtttattt	pWX1040
oWX2805	gccctgcgggacatcgcggaataattaccaatgcttaatcagtgaggc	pWX1040
oWX2806	gcctcactgattaagcattggtaattattccgcgatgtcccgcagggc	pWX1040
oWX2807	gctagcgaattcgtggatccagataagaactgctggccgttcatcgc	pWX1040
oWX2847	ctcacggtaaatggtcgagcgatg	sequencing
oWX2848	tcgatgaggatggccggatgagac	sequencing
oWX2849	gcttgatctcacggtaaatggtcg	sequencing
oWX2850	tggttatgctctcgacgagcgctc	sequencing
oWX2912	atcgagtcaaggaagacagcatatgggaaaagatcttgtacctccg	pWX1076
oWX2913	catgtttccacctcctttcaatggtcacccgccaaacttcccgctgcg	pWX1076
oWX2914	ccattgaaaggaggtggaaacatgatggacgacagcaacgatctcttc	pWX1076
oWX2915	atggcagcagtttttccgctggc	pWX1076
oWX2920	tcgaatttgctttcgaattgctagctcaaatatccagattatccgcg	sequencing
oWX2922	gcaatcaacttgccctgggtctcc	sequencing
oWX2923	atcggtaagttccacccgcatggc	sequencing
oWX2924	agcttgtcggcgccgagcgtatag	sequencing
oWX2925	gctgcgggtttcggctgcacattc	sequencing
oWX2926	ttcagggatttcgccatcggccgg	sequencing
oWX2927	gcagcggcaggatggcctgattg	sequencing
oWX2936	ttatcgagtcaaggaagacagcatatgacgcaaacttataacgctgatg	pWX1080
oWX2939	cgaatttgctttcgaattgctagcttactcttcgctatcaccgctgc	pWX1080
oWX2940	tctgcatgcaggcggtaaattctc	sequencing
oWX2941	cagacgaaagagcgtctctcttcg	sequencing
oWX2942	cagacgaaagagcgtctctcttcg	sequencing
oWX2943	accgcataacctgcgtgaagtggc	sequencing
oWX2944	actgcgtcatcttgccaaactgg	sequencing
oWX2945	agtgatctgccgcagctgtcgaag	sequencing
F1 (oWX2212)	tgtctgggttctggaattcgacgc	check configuration
R1 (oWX2213)	aggttccgtggtatagttgtaggc	check configuration
F2 (oWX2214)	cttgatccagagtgatttcgacgc	check configuration
R2 (oWX2215)	ccttgtgaacaacgcctttgaccc	check configuration

Table S4. Oligonucleotides used in this study. Related to STAR Methods.

Supplemental References

S1. Figueroa-Cuilan, W., Daniel, J.J., Howell, M., Sulaiman, A., and Brown, P.J. (2016). Mini-Tn7 Insertion in an Artificial attTn7 Site Enables Depletion of the Essential Master Regulator CtrA in the Phytopathogen Agrobacterium tumefaciens. Appl Environ Microbiol *82*, 5015-5025. 10.1128/AEM.01392-16.

S2. Morton, E.R., Merritt, P.M., Bever, J.D., and Fuqua, C. (2013). Large deletions in the pAtC58 megaplasmid of Agrobacterium tumefaciens can confer reduced carriage cost and increased expression of virulence genes. Genome Biol Evol *5*, 1353-1364. 10.1093/gbe/evt095.

S3. Farrand, S.K., and Dessaux, Y. (1986). Proline biosynthesis encoded by the noc and occ loci of Agrobacterium Ti plasmids. J Bacteriol *167*, 732-734. 10.1128/jb.167.2.732-734.1986.

S4. Fuqua, C., Burbea, M., and Winans, S.C. (1995). Activity of the Agrobacterium Ti plasmid conjugal transfer regulator TraR is inhibited by the product of the traM gene. J Bacteriol *177*, 1367-1373. 10.1128/jb.177.5.1367-1373.1995.

S5. Robalino-Espinosa, J.S., Zupan, J.R., Chavez-Arroyo, A., and Zambryski, P. (2020). Segregation of four Agrobacterium tumefaciens replicons during polar growth: PopZ and PodJ control segregation of essential replicons. Proc Natl Acad Sci U S A *117*, 26366-26373. 10.1073/pnas.2014371117.

S6. Ellis, J.G., Kerr, A., Tempe, J., and Petit, A. (1979). Arginine catabolism: a new function of both octopine and nopaline Ti-plasmids of Agrobacterium. Mol Gen Genet *173*, 263-269. 10.1007/BF00268636.

S7. Luo, Z.Q., Clemente, T.E., and Farrand, S.K. (2001). Construction of a derivative of Agrobacterium tumefaciens C58 that does not mutate to tetracycline resistance. Mol Plant Microbe Interact *14*, 98-103. 10.1094/MPMI.2001.14.1.98.

S8. Morton, E.R., Platt, T.G., Fuqua, C., and Bever, J.D. (2014). Non-additive costs and interactions alter the competitive dynamics of co-occurring ecologically distinct plasmids. Proc Biol Sci *281*, 20132173. 10.1098/rspb.2013.2173.

S9. Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B., Goldman, B.S., Cao, Y., Askenazi, M., Halling, C., et al. (2001). Genome sequence of the plant pathogen and biotechnology agent Agrobacterium tumefaciens C58. Science *294*, 2323-2328. 10.1126/science.1066803.

S10. Chesnokova, O., Coutinho, J.B., Khan, I.H., Mikhail, M.S., and Kado, C.I. (1997). Characterization of flagella genes of Agrobacterium tumefaciens, and the effect of a bald strain on virulence. Mol Microbiol *23*, 579-590. 10.1046/j.1365-2958.1997.d01-1875.x.

S11. Wu, C.F., Santos, M.N.M., Cho, S.T., Chang, H.H., Tsai, Y.M., Smith, D.A., Kuo, C.H., Chang, J.H., and Lai, E.M. (2019). Plant-Pathogenic Agrobacterium tumefaciens Strains Have Diverse Type VI Effector-Immunity Pairs and Vary in In-Planta Competitiveness. Mol Plant Microbe Interact *32*, 961-971. 10.1094/MPMI-01-19-0021-R.

S12. Anand, A., Uppalapati, S.R., Ryu, C.M., Allen, S.N., Kang, L., Tang, Y., and Mysore, K.S. (2008). Salicylic acid and systemic acquired resistance play a role in attenuating crown gall disease caused by Agrobacterium tumefaciens. Plant Physiol *146*, 703-715. 10.1104/pp.107.111302.

S13. Watson, B., Currier, T.C., Gordon, M.P., Chilton, M.D., and Nester, E.W. (1975). Plasmid required for virulence of Agrobacterium tumefaciens. J Bacteriol *123*, 255-264. 10.1128/jb.123.1.255-264.1975.

S14. Liu, P., and Nester, E.W. (2006). Indoleacetic acid, a product of transferred DNA, inhibits vir gene expression and growth of Agrobacterium tumefaciens C58. Proc Natl Acad Sci U S A *103*, 4658-4662. 10.1073/pnas.0600366103.

S15. Ren, Z., Liao, Q., Karaboja, X., Barton, I.S., Schantz, E.G., Mejia-Santana, A., Fuqua, C., and Wang, X. (2022). Conformation and dynamic interactions of the multipartite genome in Agrobacterium tumefaciens. Proc Natl Acad Sci U S A *119*. 10.1073/pnas.2115854119.

S16. Grangeon, R., Zupan, J., Jeon, Y., and Zambryski, P.C. (2017). Loss of PopZ At activity in Agrobacterium tumefaciens by Deletion or Depletion Leads to Multiple Growth Poles, Minicells, and Growth Defects. mBio *8*. 10.1128/mBio.01881-17.

S17. Ren, Z., Liao, Q., Barton, I.S., Wiesler, E.E., Fuqua, C., and Wang, X. (2022). Centromere Interactions Promote the Maintenance of the Multipartite Genome in Agrobacterium tumefaciens. mBio, e0050822. 10.1128/mbio.00508-22.

S18. Fellay, R., Frey, J., and Krisch, H. (1987). Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. Gene *52*, 147-154. 10.1016/0378-1119(87)90041-2.

S19. Hinz, A.J., Larson, D.E., Smith, C.S., and Brun, Y.V. (2003). The Caulobacter crescentus polar organelle development protein PodJ is differentially localized and is required for polar targeting of the PleC development regulator. Mol Microbiol *47*, 929-941. 10.1046/j.1365-2958.2003.03349.x.

S20. Khan, S.R., Gaines, J., Roop, R.M., 2nd, and Farrand, S.K. (2008). Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. Appl Environ Microbiol *74*, 5053-5062. 10.1128/AEM.01098-08.

S21. Choi, Y.J., Morel, L., Le Francois, T., Bourque, D., Bourget, L., Groleau, D., Massie, B., and Miguez, C.B. (2010). Novel, versatile, and tightly regulated expression system for Escherichia coli strains. Appl Environ Microbiol *76*, 5058-5066. 10.1128/AEM.00413-10.

S22. Madabhushi, R., and Marians, K.J. (2009). Actin homolog MreB affects chromosome segregation by regulating topoisomerase IV in Escherichia coli. Mol Cell *33*, 171-180. 10.1016/j.molcel.2009.01.001.