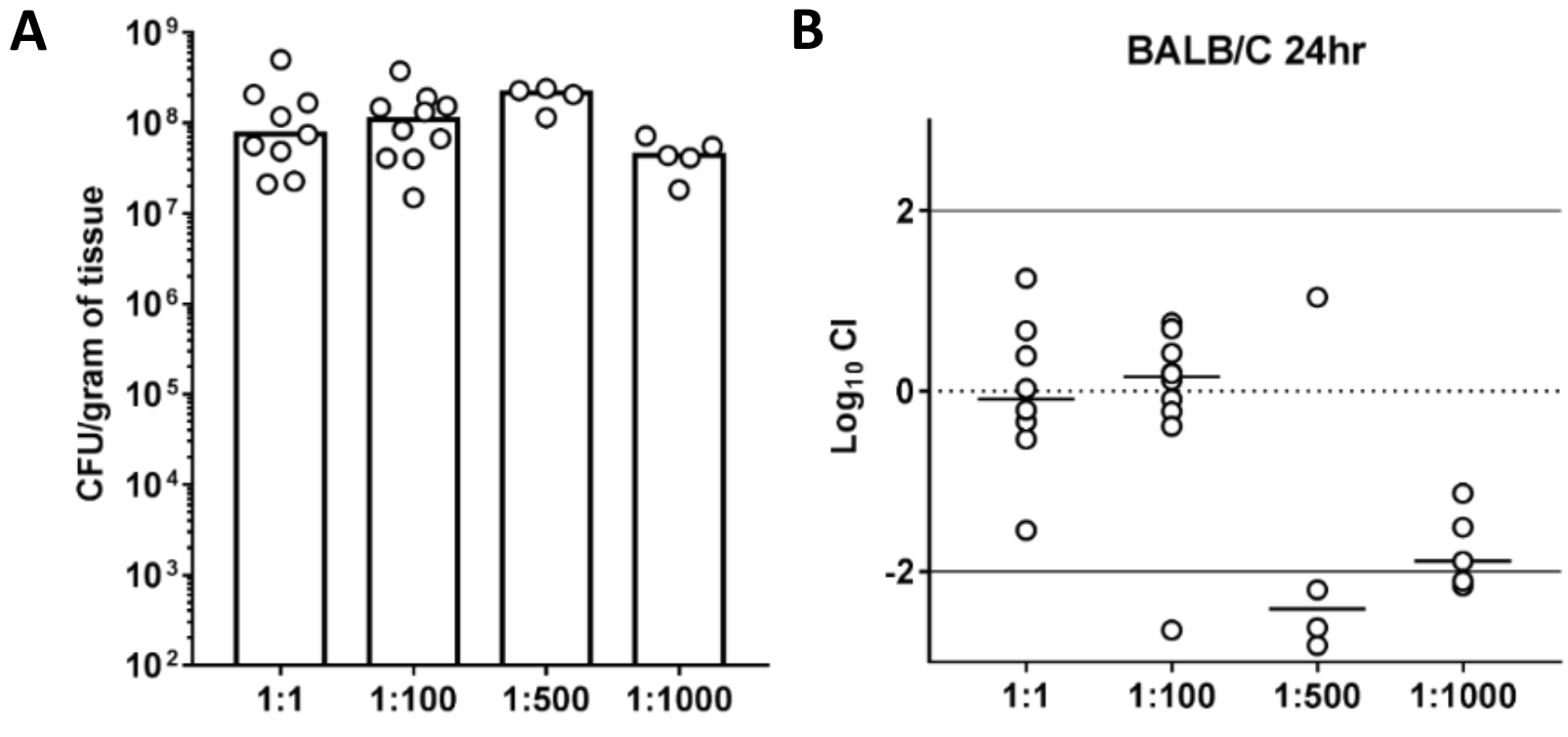
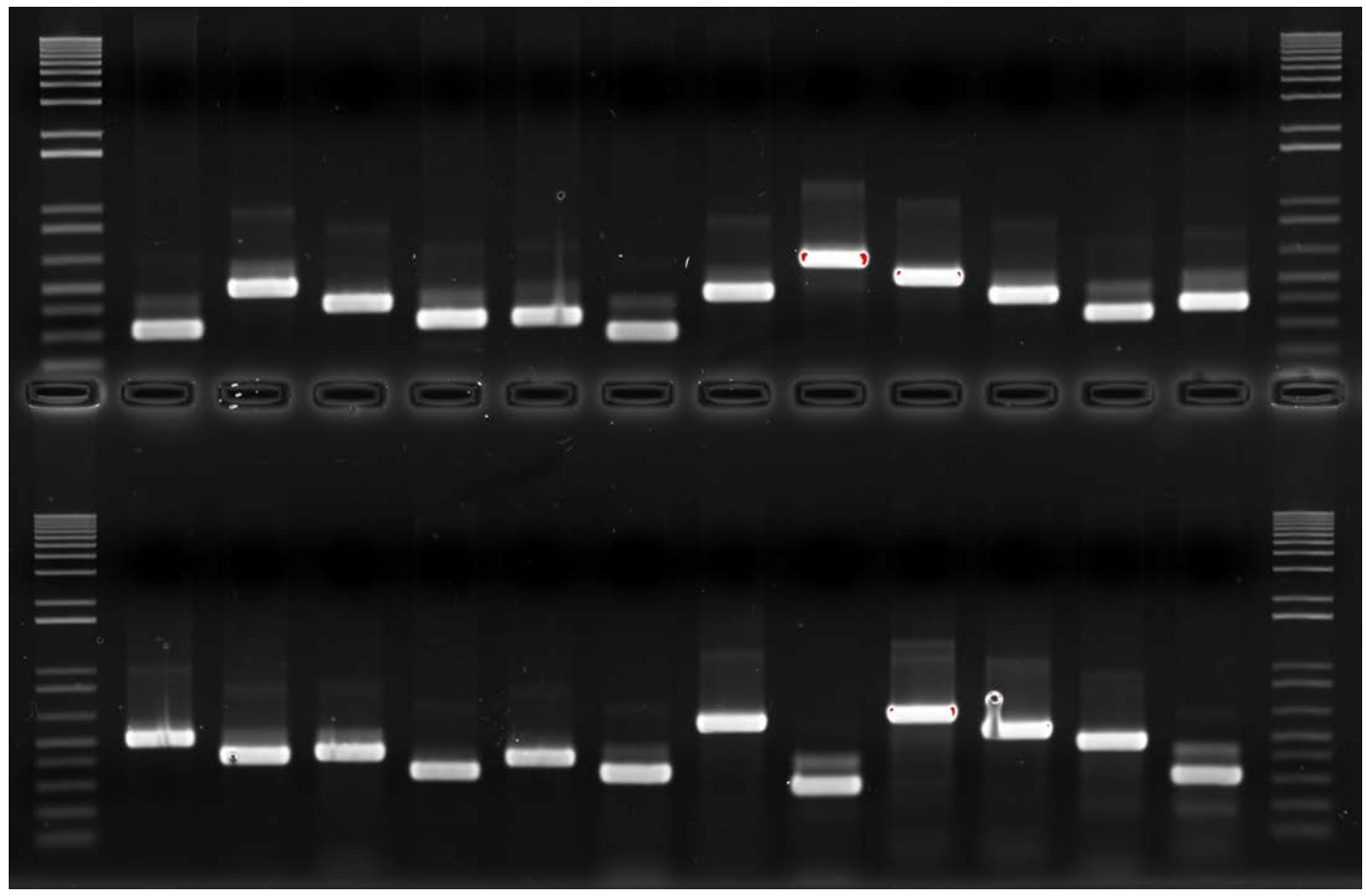
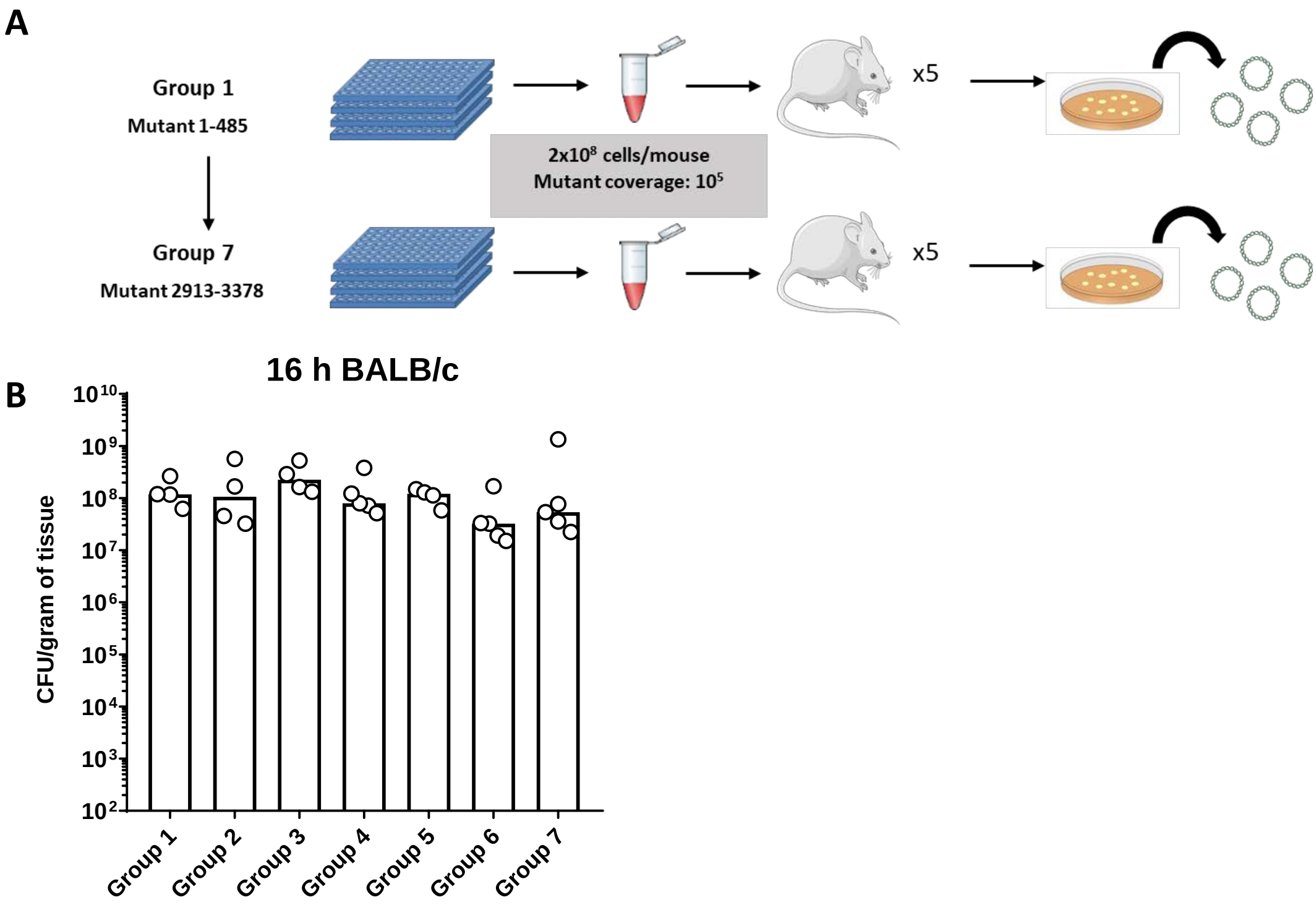


Supplement Figure 1. Characterization of the bottleneck during ascending UTI in the mouse model. To assess the bottleneck of infection during UTI, wild-type *E. coli* CFT073 and a non-attenuated mutant, Δ*araF*::Kan, were used for differential inoculum. If there is no spontaneous loss during infection, the calculated $\log_{10}$ CI will be zero. BALB/c mice were infected with either 1:1, 1:100, 1:500, or 1:1000 ratio of mutant to wild-type. (A) Mice were infected for 24 hours and bladders were harvested to enumerate bacterial CFU per gram of tissue. (B) The competitive index was calculated for each individual inoculum. A median of 0 would indicate no bottleneck effect, only 1:1 and 1:100 ratios were within the accepted range. This would indicate that only 100 different mutants could be tested at 24 hours. To increase the number of mutants that can be tested, the time of infection was decreased. (C) Mice were infected for 16 hours and bladders were harvested to enumerate CFU for each individual mouse. (D) In the 16 hour infection model, up to 500 unique mutants can be tested without spontaneous loss, as indicated by a $\log_{10}$ CI median of -0.29.

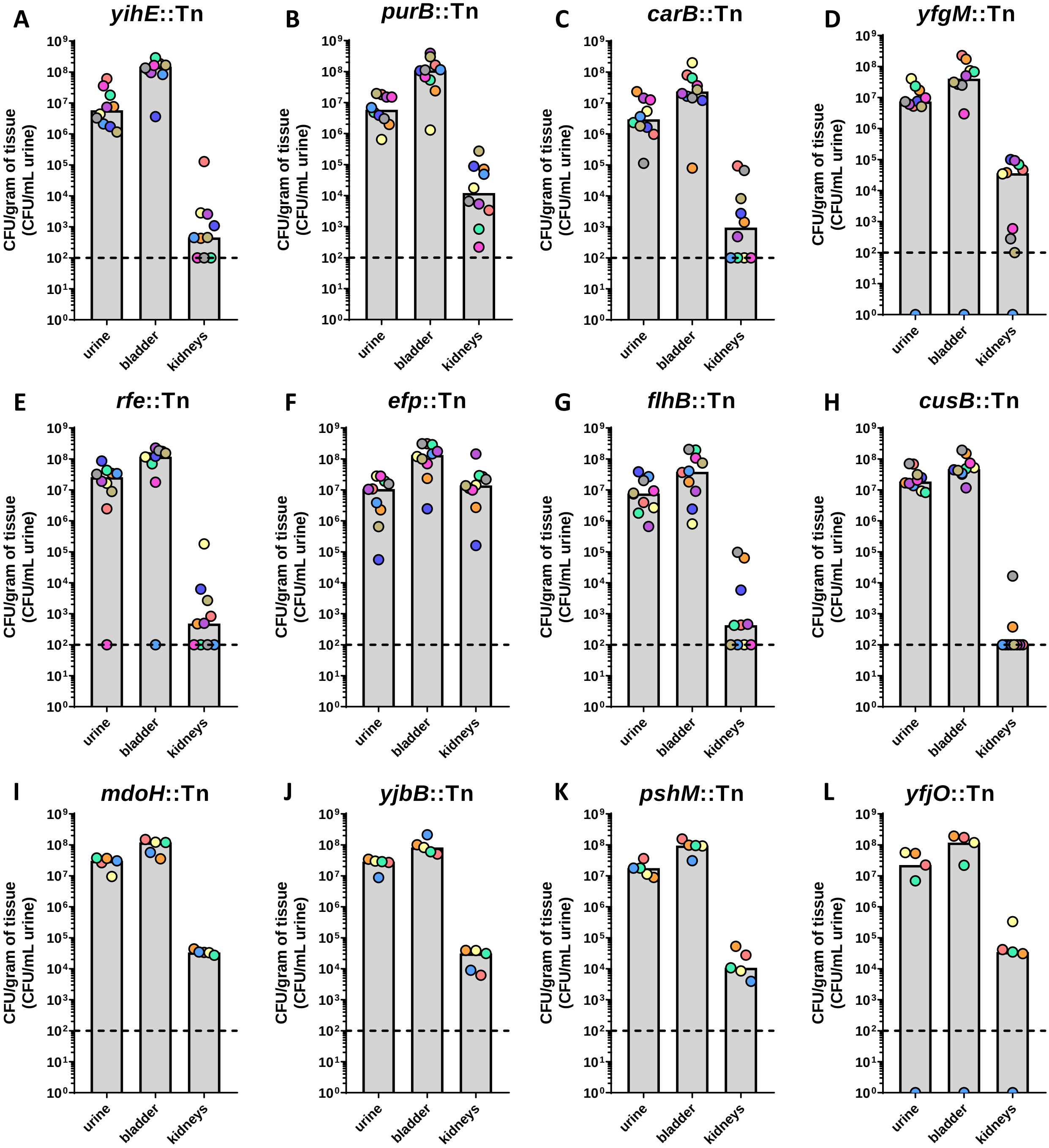


Supplement Figure 2. Positional validation of Tn insertion mutants by PCR. The positional assignments of 24 Tn mutants by CP-CSeq were verified by PCR with primers hybridizing to the transposon inverted repeat sequence P7 and a gene specific primer. All the PCR products showed the expected fragment sizes. The transposon TA insertion site coordinates, corresponding well and plate positions and specific primers can be found in Supplementary Table 2.

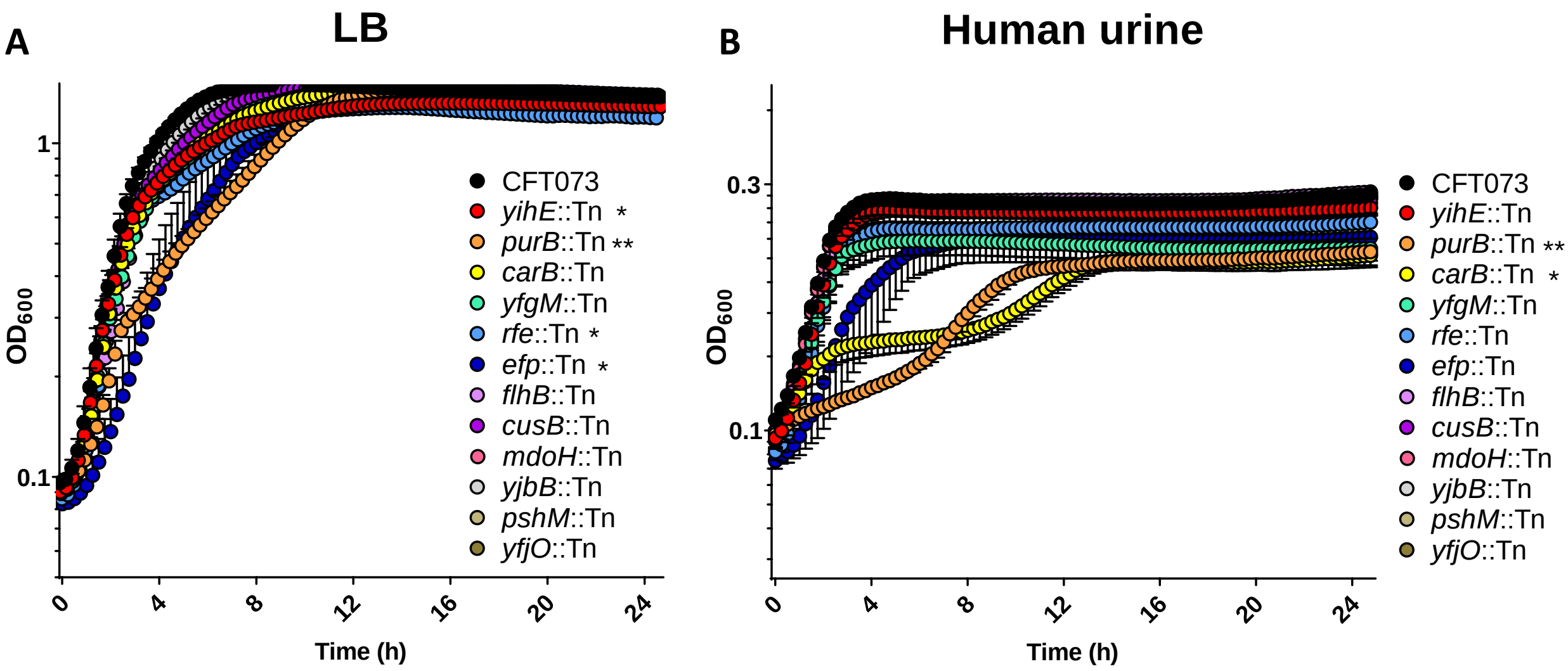


Supplemental Figure 3. Infection model and design for testing the ordered library.

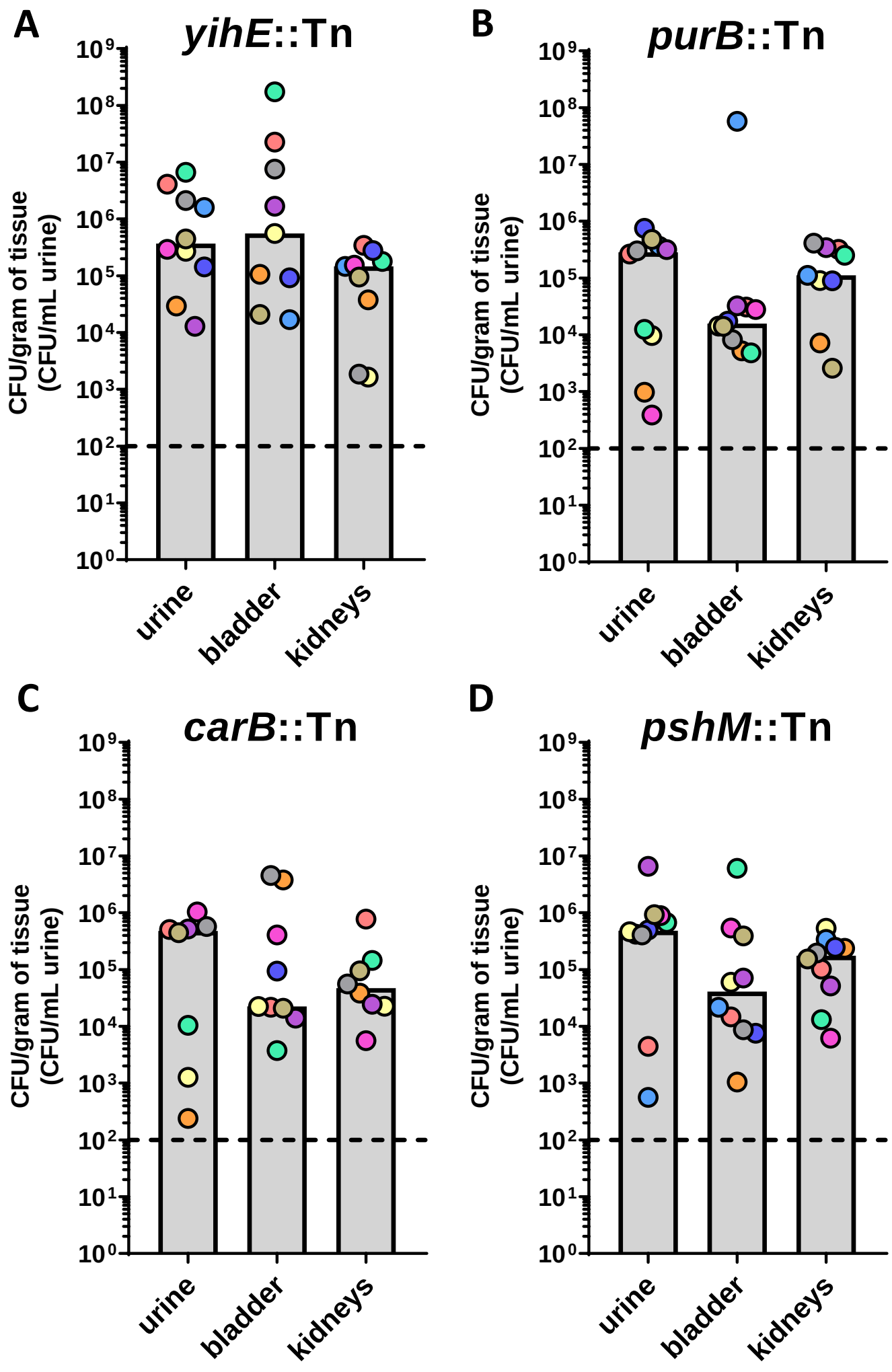
The bottleneck during a 16 hour BALB/c infection was determined to be 500 mutants, therefore the 3373 transposon mutants collected to make the condensed ordered library were grouped into 7 pools of ~485 mutants. (A) Each pool of $2x10^8$ CFU was transurethrally inoculated in the bladders of 5 mice. Bladders were harvested at 16 hpi. Total homogenates were plated onto 10 LB agar plates and incubated overnight. Bacterial lawns were collected with a sterile swab and gDNA was extracted for sequencing. [Images from Medical Servier Art] (B) Bacterial burden was determined for each individual mouse within an infection pool and plotted as CFU/gram with the median shown.



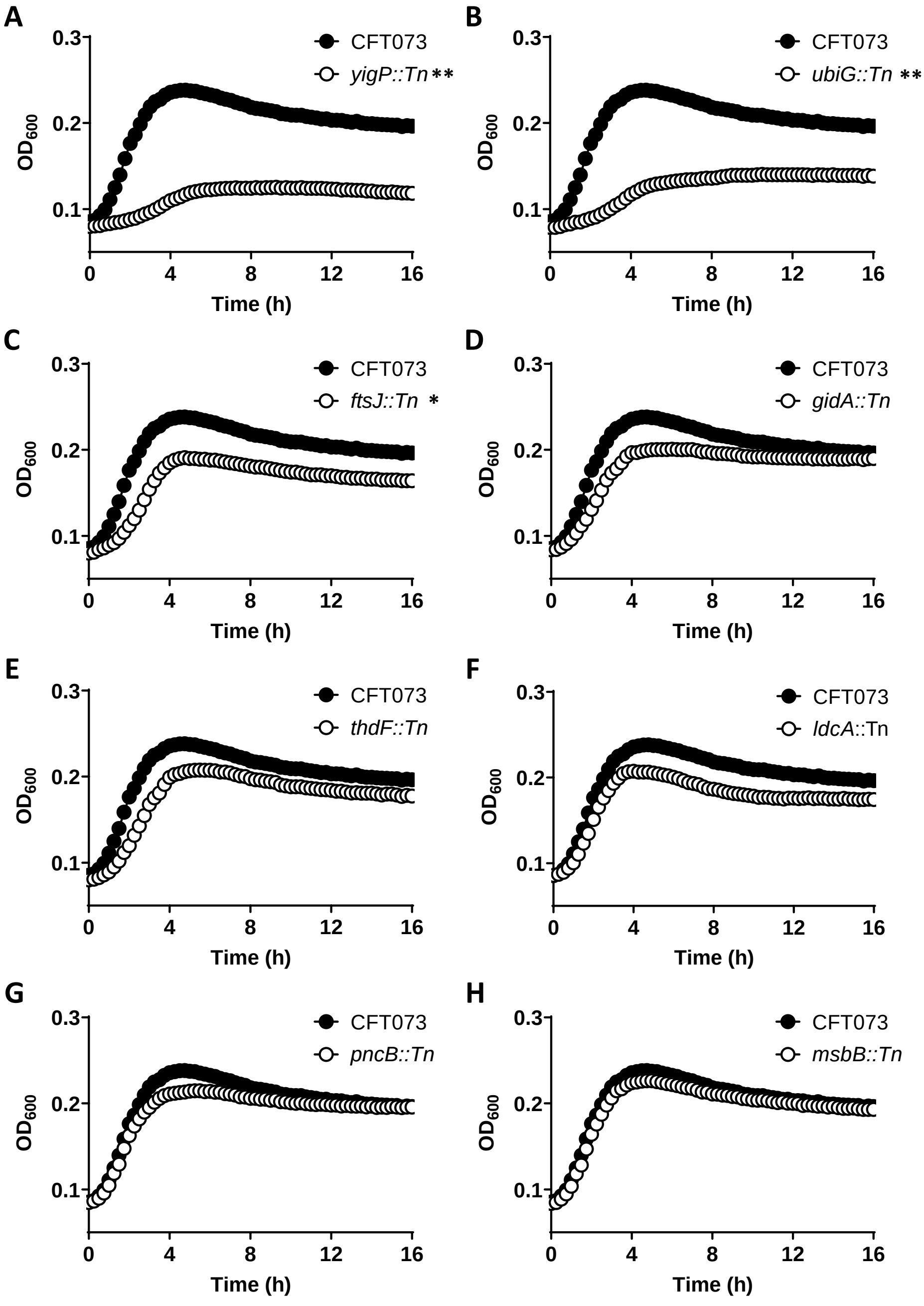
Supplement Figure 4. Bacterial burden for *in vivo* Tn-seq validation experiments. (A-L) Total CFU per gram of tissue or mL of urine is plotted for each individual mouse. Colors coordinate to Fig. 3, with the bar height indicating the median. Dashed lines indicate the limit of detection for CFU.



Supplement Figure 5. Growth of wild-type *E. coli* CFT073 and mutants from *in vivo* co-challenge experiments in LB broth and human urine. CFT073 and all transposon mutants used to inoculate in the mouse model were tested for growth in (A) LB medium or (B) human urine. Cultures were grown shaking at 37°C in media for 24 hours, with OD_{600} measurements taken every 15 minutes. The mean of three independent trials is plotted by a single dot at every time point, error bars display SEM. All mutants are plotted with wild-type CFT073 for comparison. Area under the curve (AUC) was calculated for each trial and compared to the wild-type AUC using Kruskal–Wallis one-way ANOVA to determine statistical power. *, $P<0.05$; **, $P<0.01$.

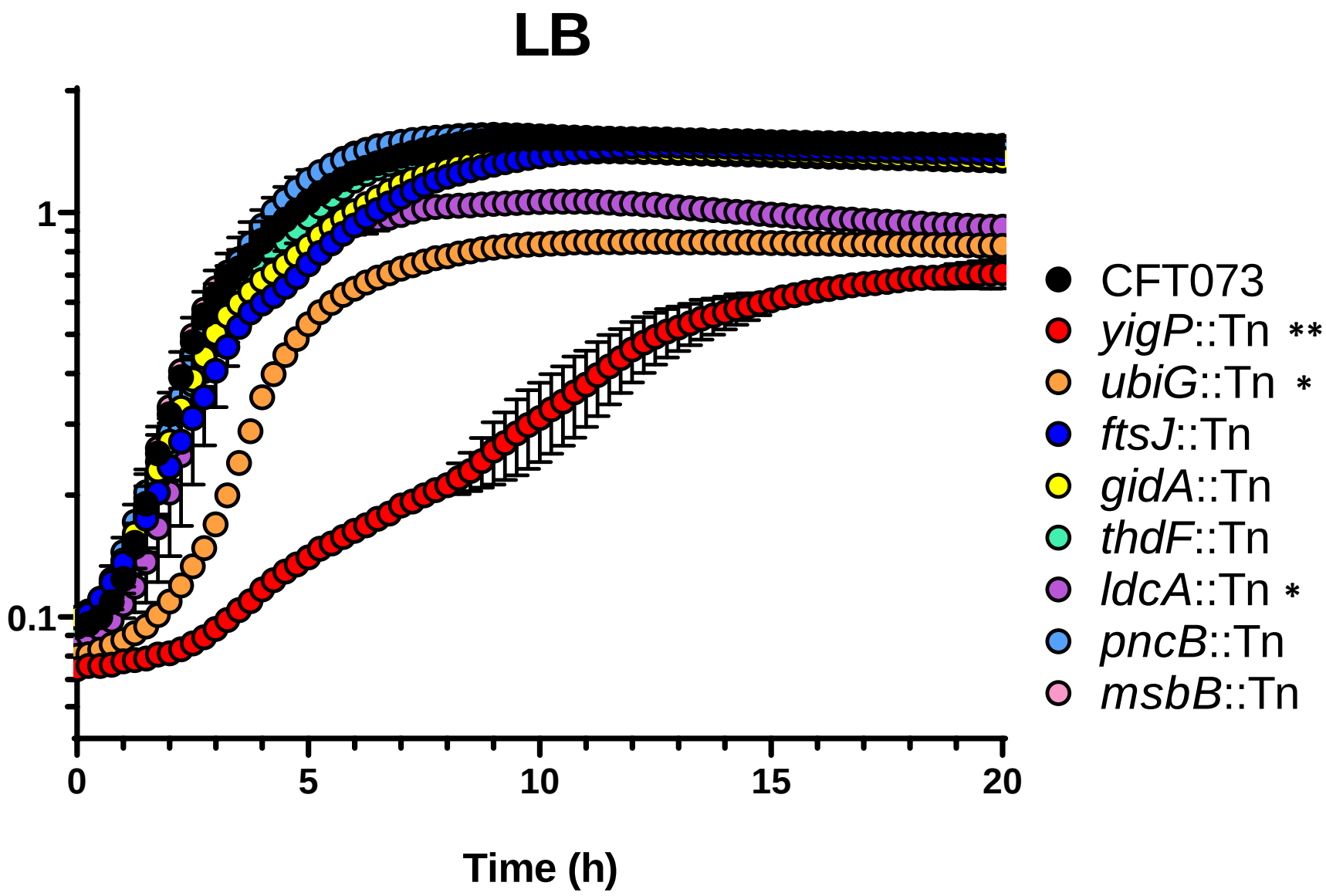


Supplement Figure 6. CFU 48hr CBA/J UTI model. (A-D) Total CFU per gram of tissue (or mL of urine) is plotted for each individual mouse with the bar indicating the median. Colors of the individual mouse data points correlate to those in Fig. 4. Dashed lines indicate the limit of detection for CFU.

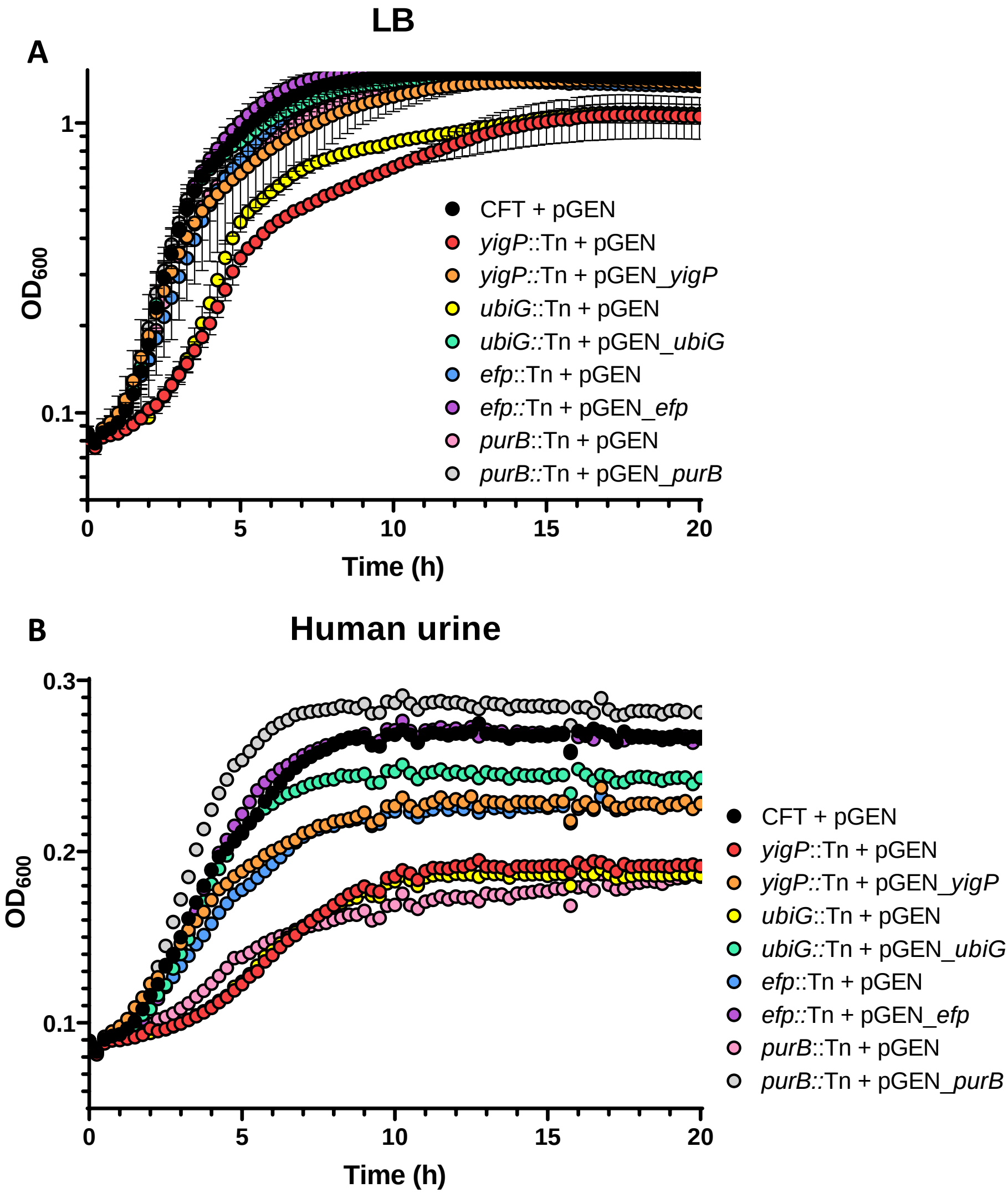


Supplemental Figure 7. Validation of human urine fitness factors from *in vitro* Tn-seq.

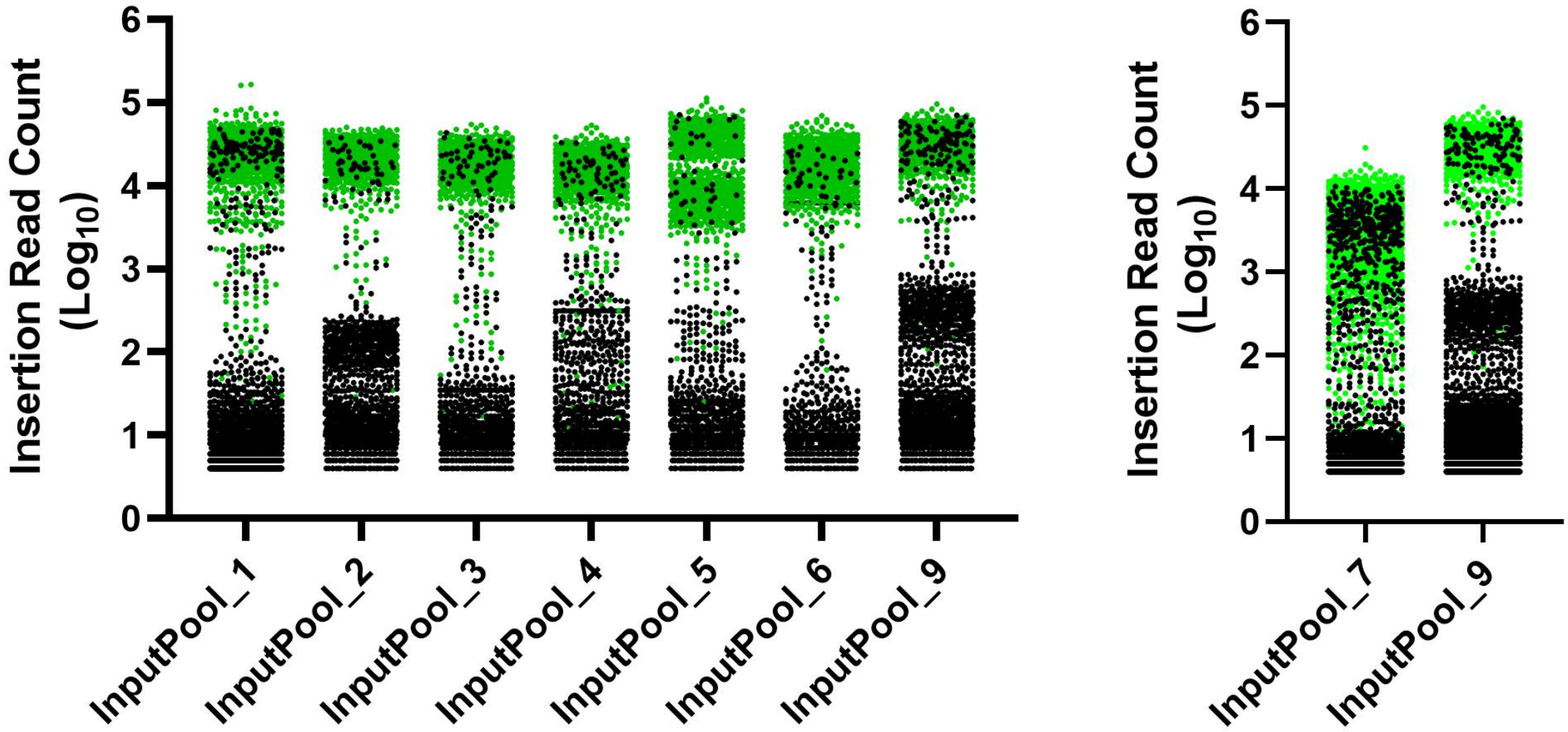
CFT073 and eight chosen mutants with growth defects as defined by *in vitro* Tn-seq analyzed in human urine. Cultures were grown statically at 37°C in human urine for 16 hours, with OD_{600} measurements taken every 15 minutes. (A-H) The mean of three independent trials is plotted by a single dot at every time point. Each mutant is individually plotted with wild-type CFT073 for comparison. SEM bars are not visible due to the symbol size. Area under the curve (AUC) was calculated for each trial and compared to the wild-type AUC using Kruskal–Wallis one-way ANOVA to determine statistical power. *, $P<0.05$; **, $P<0.01$.



Supplement Figure 8. *In vitro* human urine fitness factors grown in LB medium. Cultures were incubated shaking at 37ºC in LB medium for 20 hours, with OD_{600} measurements taken every 15 minutes. The mean of three independent trials is plotted by a single dot at every time point, error bars indicate SEM. All mutants are plotted with wild-type CFT073 for comparison. Area under the curve (AUC) was calculated for each trial and compared to the wild-type AUC using Kruskal–Wallis one-way ANOVA to determine statistical power. *, $P<0.05$; **, $P<0.01$.



Supplement Figure 9. Genetic complementation of select transposon mutants with *in vitro* growth defects. CFT073 containing empty vector pGEN and select transposon mutants with previously confirmed growth defects were tested for complementation by growth in (A) LB medium or (B) human urine. Transposon strains containing either the empty vector pGEN or pGEN encoding the mutated gene for complementation *in trans*. Cultures were grown shaking at 37^{o}C in media for 20 hours, with OD_{600} measurements taken every 15 minutes. The mean of three independent trials is plotted by a single dot at every time point. Error bars represent SEM, bars are not visible on human urine data due to symbol size.



Supplement Figure 10. Plot of insertion read coverage of each mapped insertion at TA sites present in input samples. Green dots represent expected insertions at TA sites to be found in input samples and black dots represent unexpected insertions. Both populations are separated by a gap if coverage where expected insertions show the highest coverages. The median of insertions read count of those putative unexpected insertions was very low in all the samples (from 6 up to 47 reads) indicating that those insertions could be artefacts or noise. Only expected insertions (green dots) were analyzed by TnseqDiff package for fitness defect.

	GeneBank Annotation
Insertions	
No. of insertions (coverage >300)	8543
No. unique and traceable insertions	8105
Intergenic space hit	982
Intragenic hit	7123
Genes	
No. of traceable disrupted genes	2913
No. of untraceable disrupted genes	29
No. of untagged genes	2478
% saturation	53.75%
Operons	
No. of traceable disrupted operons	876
No. of untagged operons	241
% saturation	78.04%

Supplement Table 1. Results of CP-CSeq on a 96 x 96-well transposon insertion library of *E. coli* CFT073. Distribution of both the mutated and unmutated/untraceable gene and operon fraction in the CFT073 Tn insertion library.

PCR rxn Number	Oligo Name	Sequence (5' to 3')	Plate	Well	Tn Insertion Site
1	**3455_R**	AATCCACGATGACAGGCGTT	73	2A	1826499
2	**2306_R**	ATGGACCCCACCCTGAACCA	73	2B	1051293
3	**4166_R**	GGTTGTGCTTCGGCGTTTGG	73	2D	2303127
4	**3230_R**	TAGCGGAAATTGGCTGGCGA	73	2E	1638200
5	**6443_R**	GTAAGCATCGTTGGCCCGGA	73	2F	3300493
6	**11093_R**	CAGGCGCTAACCTTGCTTGC	73	2G	4759124
7	**7794_R**	CTACCCCTTACTGCGCCTGC	73	2H	3792292
8	**10029_R**	CCGCTGCACACCAGAACAGA	85	2A	4449374
9	**5591_R**	GCGGAACTGGAACAGGCGAA	85	2B	2994975
10	**5825_R**	CTTCACGTCGTGCCTGGGAG	85	2D	3084307
11	**4915_R**	CAGTCCGCCGTAAGTCAGCA	85	2E	2688730
12	**10680_R**	CGCCTGTTCGTCACCTTCCA	85	2F	4622280
13	**8269_R**	TGAGGCGATGCAGCCAGATG	85	2G	3934565
14	**11247_R**	TCGCTTCGGCAGGGTTCTTG	85	2H	4796968
15	**6750_R**	GCGGATAGTCGCAGTGGCAT	7	5E	3404428
16	**6859_R**	ACTGGAAAGTCACAGCCCGC	7	5F	3456250
17	**10403_R**	GCAGCGGTTTTCGGTTCAGC	7	5G	505330
18	**4249_R**	GCAGCCAGGCTTCCACTTCA	7	5H	2342414
19	**2381_R**	GCTCGTTTTCGTTACGGCGG	27	3C	1083833
20	**3200_R**	TGCCACCATTGACGCCAGTT	40	4D	1621104
21	**3763_R**	TCAACTGCACGCTCCCTTCG	53	5E	2076282
22	**1461_R**	AGCGTCGGGCGCATAATCAA	66	6F	672591
23	**7569_R**	TTCGTCGATCCAGCTTCCGC	79	7G	3712329
24	**8019_R**	AGTTCGGCATCAGCGGTACG	92	8H	3867538
	P7_Fw	CCAAGCAGAAGACGGCATACG			

Supplement Table 2. Primers used for the verification of the 24 mutant locations within the ordered library.

Input pools for UTI analysis		Mapped insertions[1]					
			Insertion Read Count				
			Minimum	25% Percentile	Median	75% Percentile	Maximum
Input 1 (replic. 1)	Expected:	94.45 % (460 of 487)	30	16064	25009	32921	161064
	Unexpected:	800	4	5	6	14	47355
Input 1 (replic. 2)	Expected:	94.45 % (460 of 487)	26	15578	25135	33578	165756
	Unexpected:	836	4	5	7	15	48169
Input 2 (replic. 1)	Expected:	94.62 % (458 of 484)	35	18067	23482	29526	50389
	Unexpected:	1009	4	7	47	133	37734
Input 2 (replic. 2)	Expected:	94.62 % (458 of 484)	29	15805	20849	26667	47610
	Unexpected:	859	4	5	8	13	32959
Input 3 (replic. 1)	Expected:	92.38 % (449 of 486)	20	15343	20870	28070	54377
	Unexpected:	565	4	5	6	12	43220
Input 3 (replic. 2)	Expected:	92.38 % (449 of 486)	17	12029	15891	21164	38080
	Unexpected:	622	4	5	7	14	32535
Input 4 (replic. 1)	Expected:	95.06 % (462 of 486)	17	12009	16178	21242	49768
	Unexpected:	662	4	6	13	86	29769
Input 4 (replic. 2)	Expected:	95.06 % (462 of 486)	13	10250	15488	20421	53489
	Unexpected:	457	4	5	7	13	32742
Input 5 (replic. 1)	Expected:	95.4 % (460 of 484)	26	4646	6662	9162	24846
	Unexpected:	427	4	4	6	24	13316
Input 5 (replic. 2)	Expected:	95.4 % (460 of 484)	112	28695	38877	49953	113026
	Unexpected:	887	4	5	6	11	71264
Input 6 (replic. 1)	Expected:	91.24% (448 of 491)	137	17094	23152	29948	61791
	Unexpected:	560	4	4	6	11	29248
Input 6 (replic. 2)	Expected:	91.24% (448 of 491)	61	9007	12642	16277	69891
	Unexpected:	299	4	4	7	16	15710
Input 7 (replic. 1)	Expected:	92.44 % (427 of 462)	12	22056	29735	39036	79784
	Unexpected:	1729	4	5	7	11	59417
Input 7 (replic. 2)	Expected:	92.44 % (427 of 462)	16	22923	31733	43503	95850
	Unexpected:	2507	4	5	8	24	70566
Input pools for Growth in Human Urine analysis							
Input 7 (replic. 1)	Expected:	93.79% (2737 of 2918)	7	3671	5109	6836	31033
	Unexpected:	714	4	5	9	1324	10691
Input 7 (replic. 2)	Expected:	91.24% (448 of 491)	5	2373	3231	4197	17939
	Unexpected:	299	4	5	24	2209	7444
Input 9 (replic. 1)	Expected:	92.44 % (427 of 462)	35	18067	23482	29526	79784
	Unexpected:	1729	4	7	47	133	37725
Input 9 (replic. 2)	Expected:	92.44 % (427 of 462)	24	15805	20849	26667	95850
	Unexpected:	2507	4	5	8	13	32912

[1] Number of insertions at TA sites identified by IN-seq showing a read coverage <3; *Expected*: Insertions selected from the CFT073 transposon mutant ordered library to be part of a given input pool. (%) represents the percentage of expected insertions that were finally identified in the corresponding input pool; *Unexpected*: Insertions that were unexpected to be found in a given input pool.

Supplement Table 3. Input pool quality analysis for expected insertion sites.

Supplemental Table 4. Strains and plasmids used in this study.

Strains	
CFT073	[HL Mobley et al., Infect Immun, May;58(5):1281-9, 1990]
CFT073 Δ*araF::*Kn	[CJ Alteri et al., PLoS Pathog, May;5(5):e1000448, 2009]
MGN-617 Auxotropic Diaminopimeli acid (DAP)	[CM Dozois et al., Infect Immun, Jul;68(7):4145-54, 2000]
Plasmids	
pSam_AraC KanR, AmpR (Backbone)	[CE Armbruster et al., PLoS Pathog, Jun 14;13(6):e1006434, 2017]