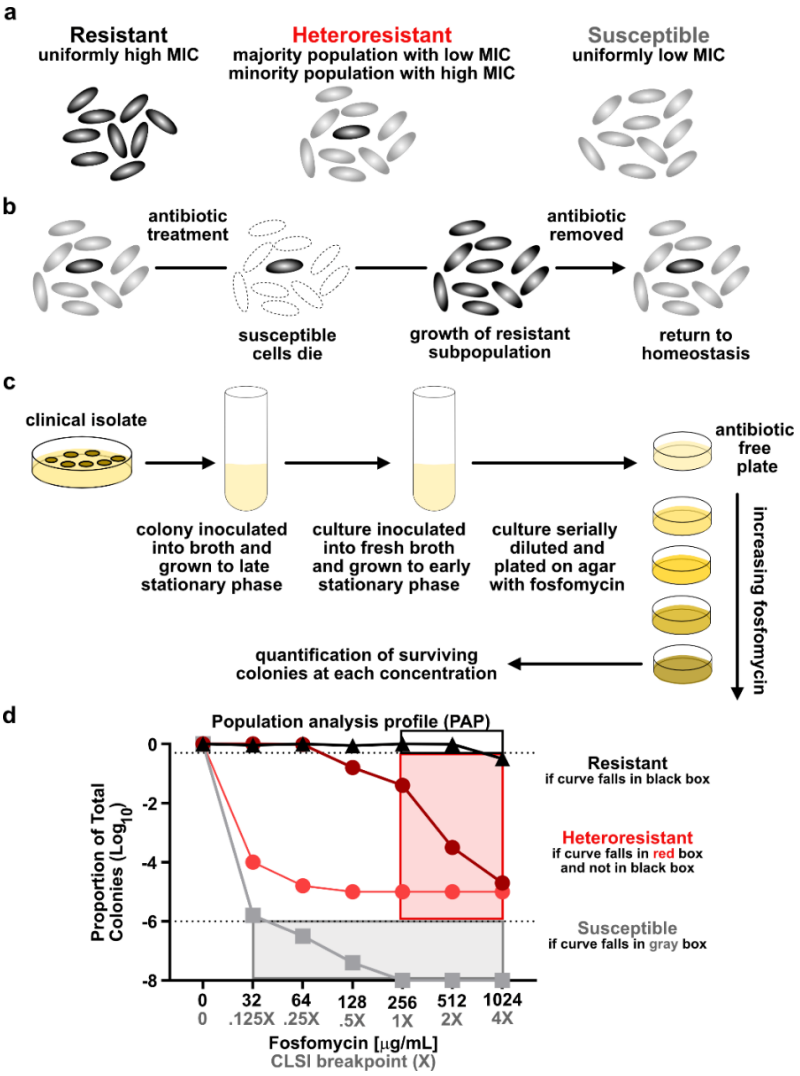
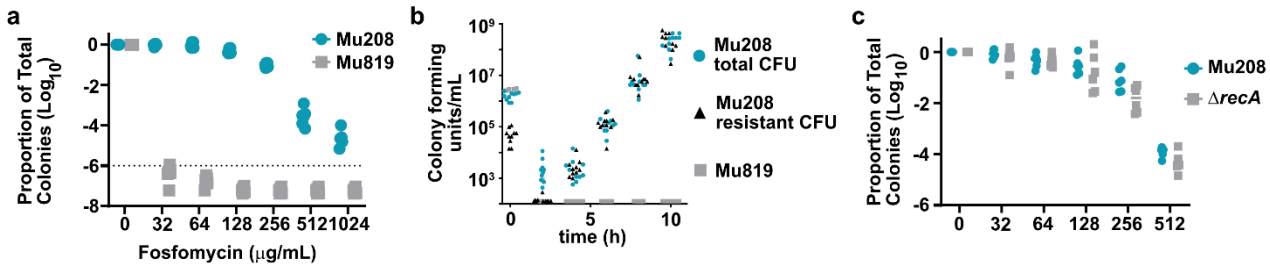


Supplementary Figure 1. Overview of heteroresistance and population analysis profile (PAP). (a) A depiction of the cells grown from a single colony of an isolate exhibiting conventional resistance, heteroresistance, or susceptibility to a given antibiotic. (b) Population dynamics of a heteroresistant isolate following antibiotic treatment. (c) Fosfomycin PAP: a clinical isolate is isolated and grown in broth, then subcultured into fresh broth and grown to early stationary phase. The culture is then serially diluted, plated on Mueller Hinton agar (MHA) with increasing concentrations of fosfomycin, and incubated. (d) The surviving colonies are enumerated and the proportion of total colonies that survive on a given concentration is calculated: Log10 ($\frac{\text{CFU/mL on antibiotic}}{\text{CFU/mL on MHA}}$). The isolate is classified as resistant if at least 50% of the total colonies grow at 1 or 2X breakpoint. An isolate is considered susceptible if less than 0.0001% (-6 logs) of the cells grow at any concentration shown. An isolate is considered heteroresistant if there is greater than 0.0001% survival at 1X and an ≥8-fold difference between the resistance of the subpopulation and resistance of the main population. As an example, if <50% of the population survives at 128 µg/mL, the resistance of the main population is 64 µg/mL. The resistance of the subpopulation that survives on 512 µg/mL is greater than 512 µg/mL, making the fold change ≥8. Along the x-axis, the breakpoints based on CLSI are shown in gray, and the corresponding concentration of fosfomycin is in black. Adapted from Choby *et al*[1] under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

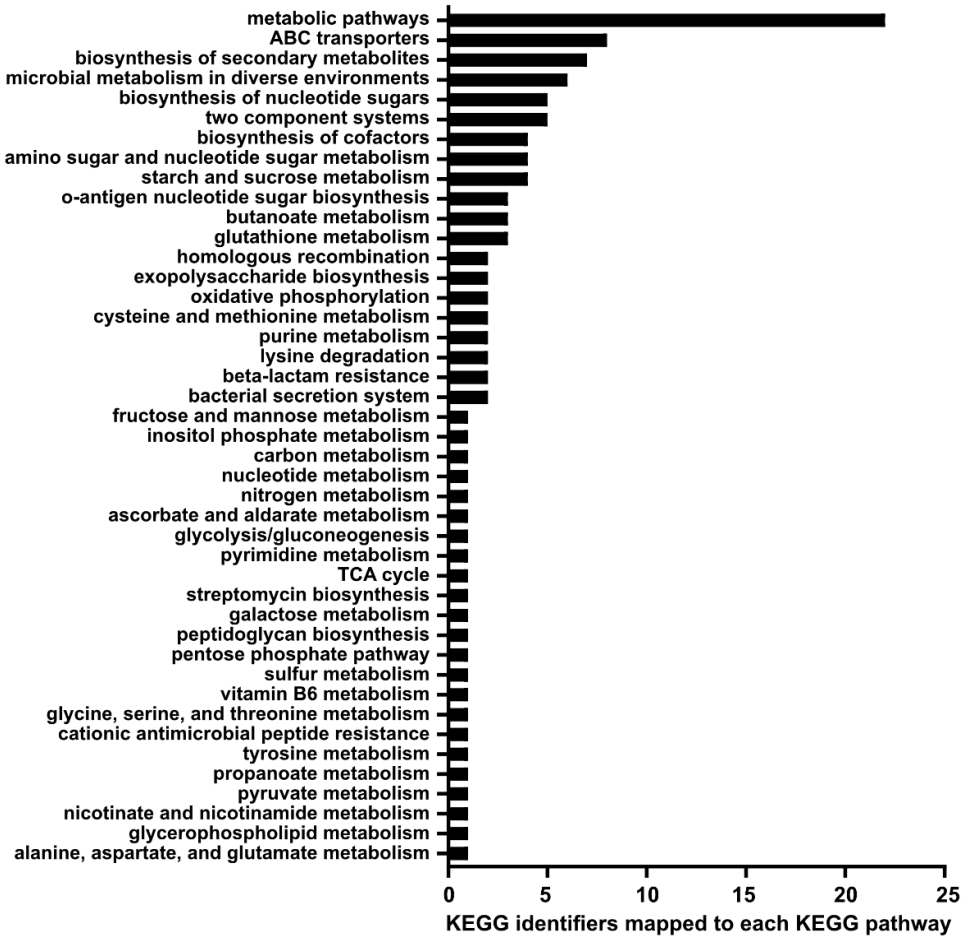


Supplementary Figure 2. Fosfomycin heteroresistance in Mu208 is *recA*-independent. (a) Alternative presentation of population analysis profile (PAP) of Mu208 and Mu819, from three independent experiments with 2 biological replicates each. (b) Alternative presentation showing each data point from Figure 1b. Time kill of Mu208 and Mu819: survival in media containing 512 µg/mL fosfomycin over time and quantification of surviving colony forming units on MHA (Mu208 total and Mu819) and MHA containing 512 µg/mL fosfomycin (resistant CFU) from two independent experiments with 5 biological replicates each for Mu208 and three replicates each for Mu819. (c) Population analysis profile (PAP) of Mu208 and the Δ*recA* deletion strain, from two independent experiments with 3 biological replicates each. Source data are provided as a Source Data file.

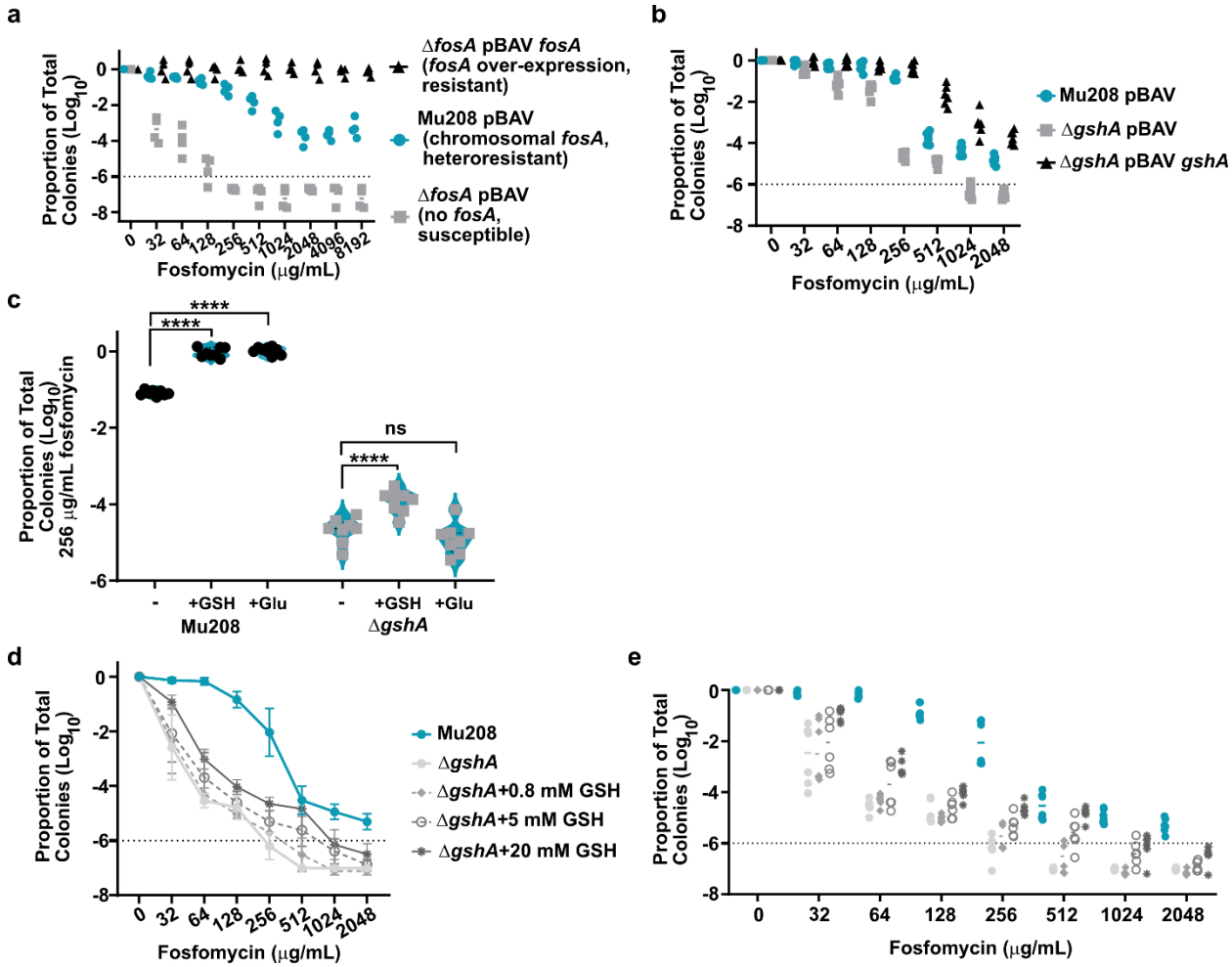
a

Colonies screened	hits	Rate
79,145	145	0.183%

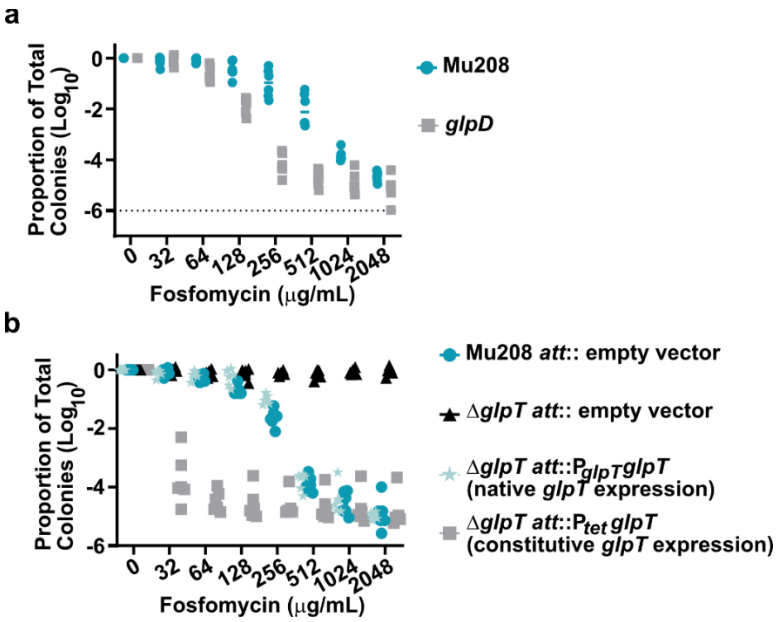
b



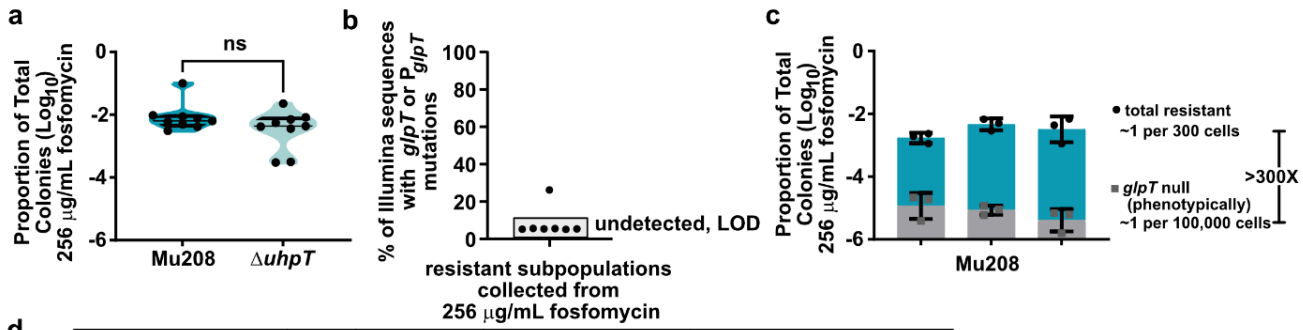
Supplementary Figure 3. Transposon screen for loss of fosfomycin resistant subpopulation identifies key role for metabolic genes. (a) Results of transposon screen. (b) The KEGG identifiers of each transposon-disrupted gene in the screen was mapped to KEGG pathways as above. A portion of the genes did not map to a KEGG identifier/pathway. Full list is provided in Supplementary Data 2



Supplementary Figure 4. FosA and glutathione synthesis support the resistant subpopulation. (a) Alternative presentation of PAP of Mu208, the Δ*fosA* deletion strain, and its complement two independent experiments with 2 biological replicates each. (b) Alternative presentation of PAP of strains Mu208, Δ*gshA* mutant and its complement two independent experiments with 3 biological replicates each. (c) Proportion of population resistant to fosfomycin following growth in Mueller Hinton broth, with addition of 20 mM glutathione (+GSH) or 40 mM L-glutamic acid (+Glu) in strains Mu208 and Δ*gshA*, from two independent experiments with 4 biological replicates each. ns indicates p=0.3374, **** indicates p<0.0001 by one-way ANOVA with Šídák's multiple comparisons test, F (5, 42) = 669.8. (d-e) PAP of Mu208 grown in Mueller-Hinton broth (MHB), or Δ*gshA* mutant grown in MHB without or with glutathione (GSH) at concentrations indicated. (e) is an alternative presentation showing each data point, (d-e) are from two independent experiments with 3 biological replicates each. (d) Data are presented as mean values +/- SD. Source data are provided as a Source Data file.



Supplementary Figure 5. GlpT expression controls subpopulation frequency. Alternative PAP presentation of fosfomycin heteroresistance in (a) Figure 2a, from two independent experiments with 3 biological replicates each and (b) Figure 2b, from two independent experiments with 3 biological replicates each. Source data are provided as a Source Data file.

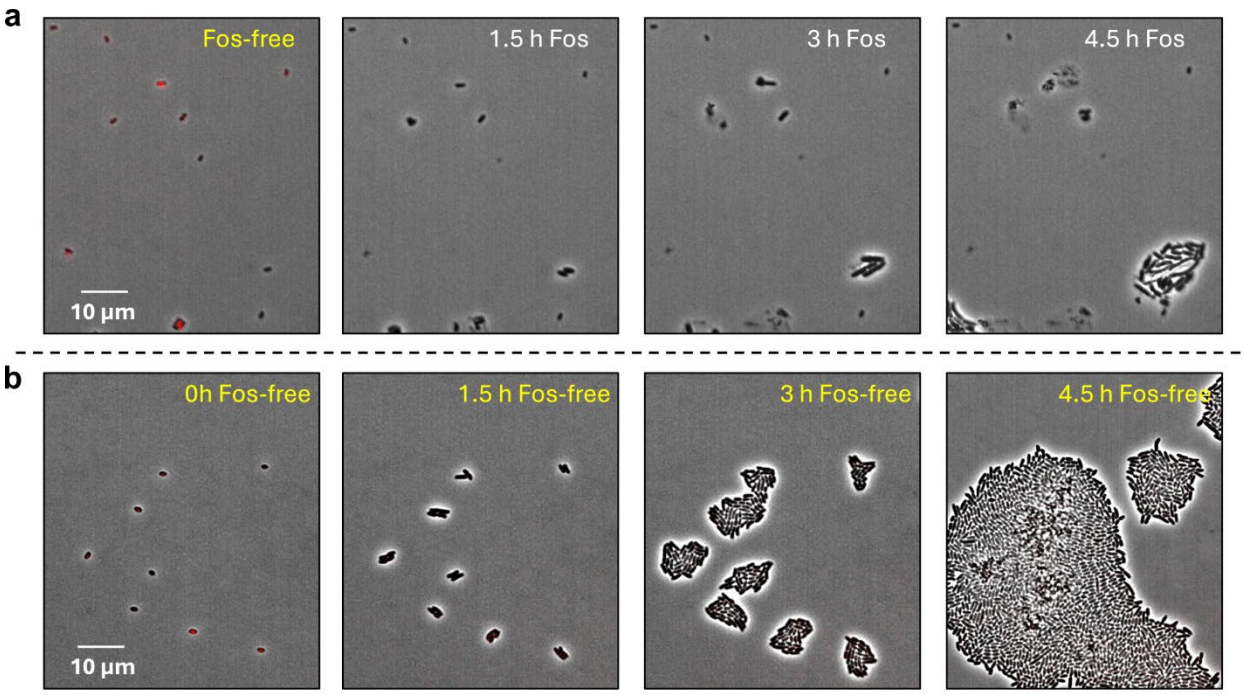


d

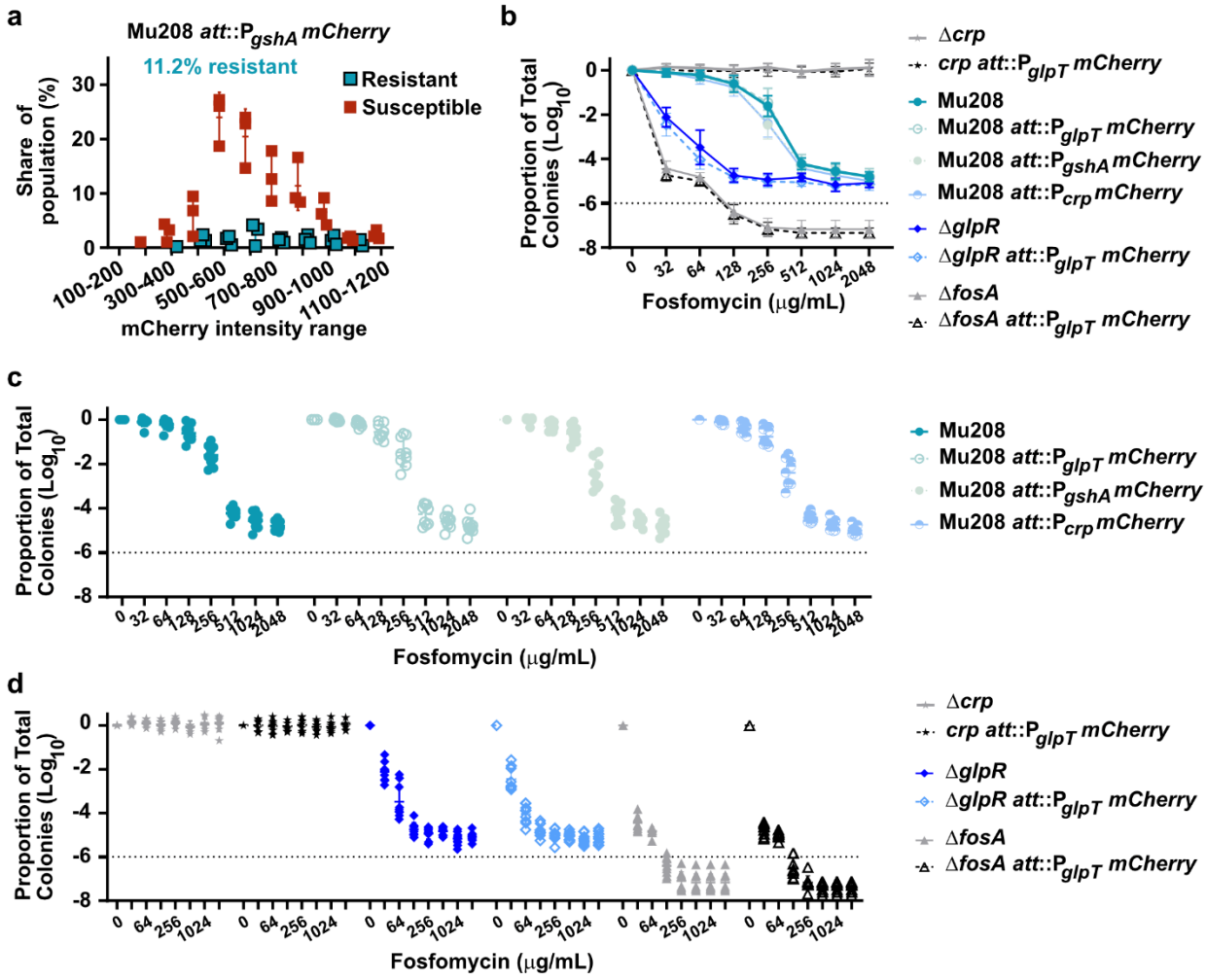
Strain	PAP panel	Fosfomycin (μg/mL)	Log10 proportion of resistant colonies	Resistant, phenotypically *glpT* null colonies*
Mu208 pBAV	1h	2048	~ -5	76.7%
Δ*gshA* pBAV	1h	256	~ -5	96.7%
Δ*glpT att*::P_{tet}*glpT*	2a	2048	~ -5	100%
Δ*glpT att*::P_{glpT}*glpT*	2a	2048	~ -5	80%
Δ*glpR*	3a	2048	~ -5	100%

*5 colonies of 6 biological replicate (30 total) were patched from agar containing fosfomycin indicated onto M9+glycerol agar

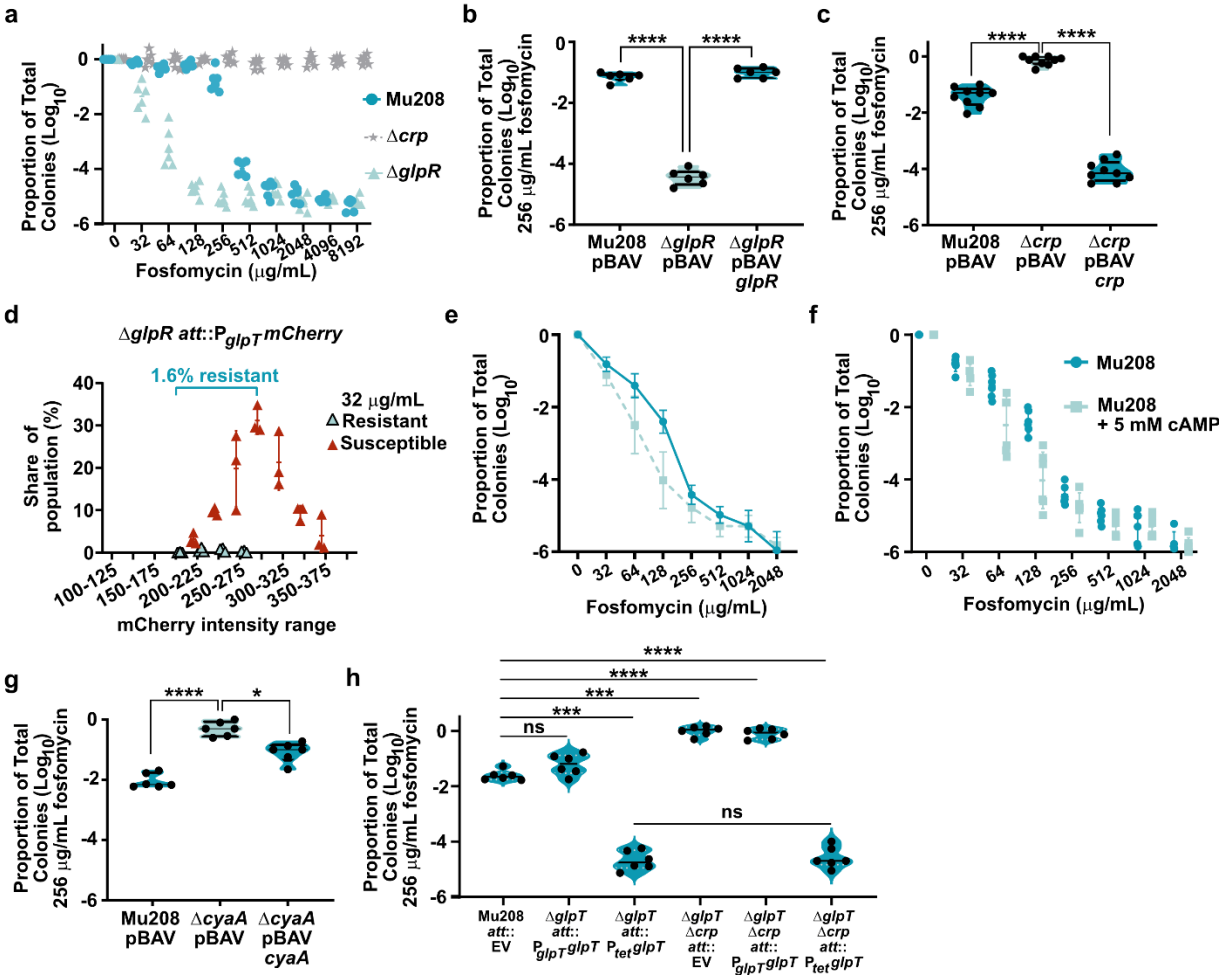
Supplementary Figure 6. Spontaneous *glpT* mutants constitute a small portion of the resistant subpopulation. (a) Proportion of colonies resistant to fosfomycin in strains Mu208 and Δ*uhpT*, encoding the hexose-phosphate transporter, from three independent experiments with 3 biological replicates each. ns indicates p=0.2294 by two-tailed t-test t=1.301, df=8. (b) P_{glpT}*glpT* mutations detected in fosfomycin resistant colonies by variant calling from Illumina sequencing. 7 colonies collected from agar containing 256 μg/mL fosfomycin were sequenced. (c) Proportion of colonies resistant to fosfomycin and then screened for loss of GlpT function in Mu208, from three independent experiments (each bar) with 3 biological replicates total. Data are presented as mean values +/- SD. (d) Fosfomycin resistant colonies from strains indicated where screened for GlpT function by assessing growth on M9+glycerol agar. Source data are provided as a Source Data file.



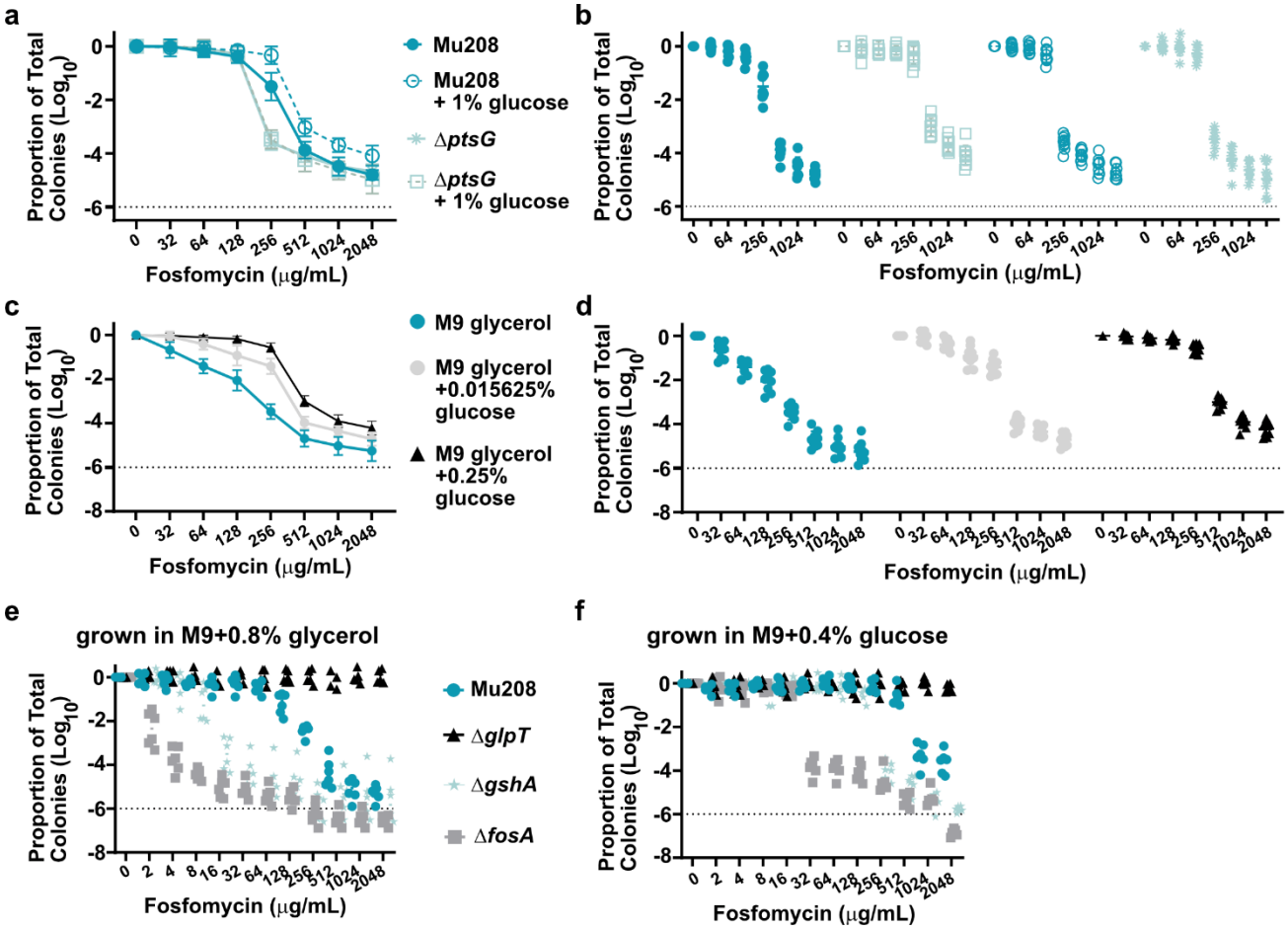
Supplementary Figure 7. Mu208 *att*:: P_{glpT}*mCherry* expression affects growth in fosfomycin but not in the absence of fosfomycin. (a) Additional representative microscopy images associated with Figure 2c. of Mu208 *att*:: P_{glpT}*mCherry* on agar pads in the absence of fosfomycin, followed by images of the time-lapse following fosfomycin addition. See also Supplementary Video (b) Growth progression of Mu208 *att*:: P_{glpT}*mCherry* in the absence of fosfomycin. (a-b) are single experiments representative of at least three similar experiments.



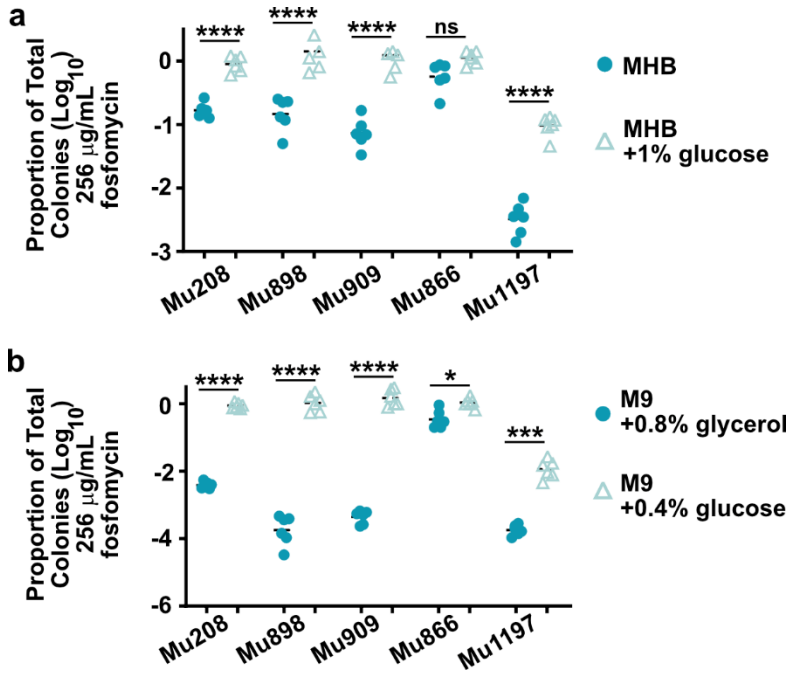
Supplementary Figure 8. P_{gshA}*mcherry* is not a predictor of survival, and reporter strains phenocopy parental strains. (a) P_{gshA}*mcherry* expression intensity among cells that survived or were killed by 128 μg/mL fosfomycin, from three independent experiments; data were determined from 700-1000 cells for each experiment. Data are presented as mean values +/- SD. (b) PAP of Mu208, mutants, and strains encoding *mCherry* reporters. Data are presented as mean values +/- SD. (c-d) Alternative PAP presentation of panel (b). (b-d) are from three independent experiments with 3 biological replicates each. Source data are provided as a Source Data file.



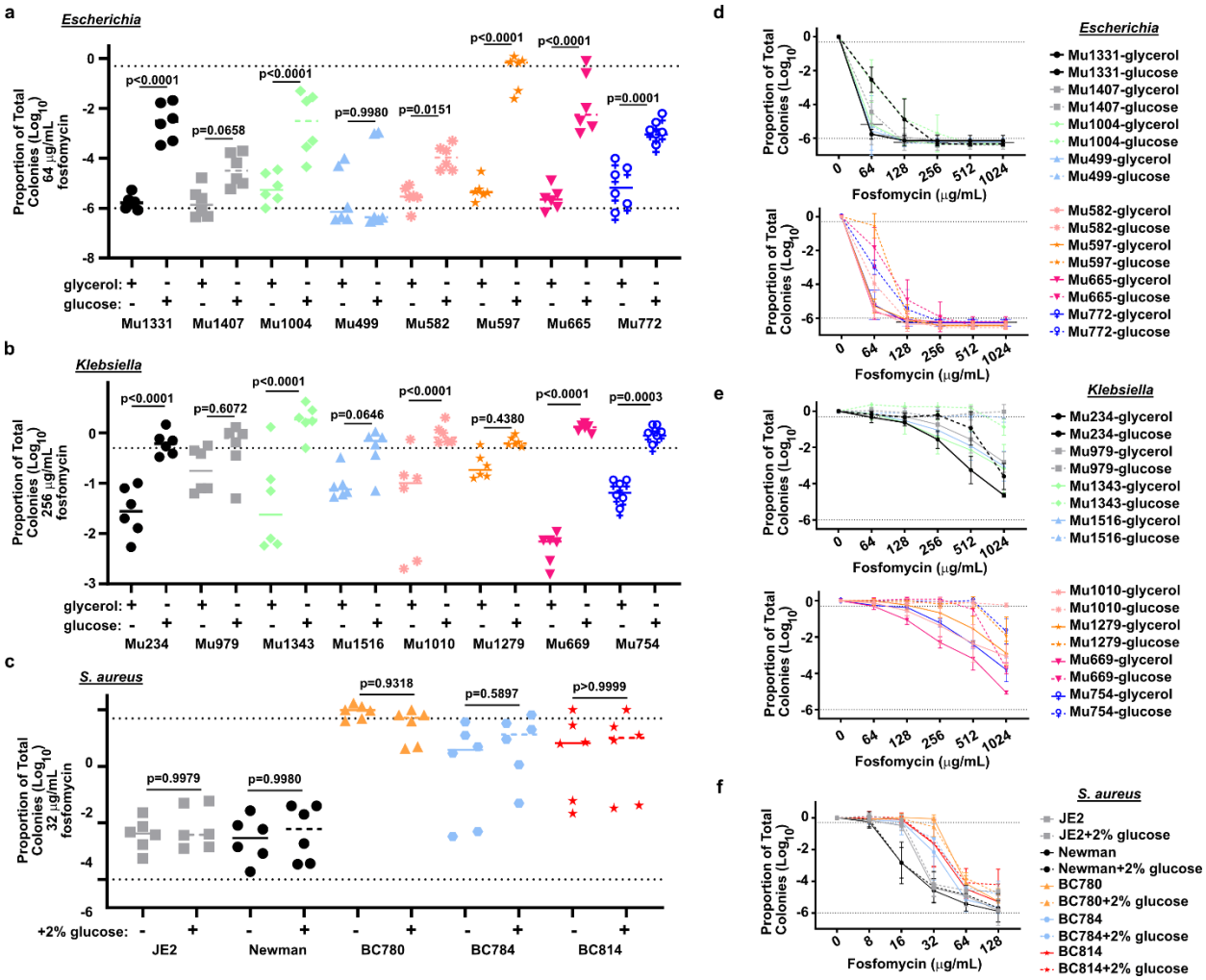
Supplementary Figure 9. Regulation of *glpT* by GlpR and CyaA/CRP controls the subpopulation. (a) Alternative presentation of population analysis profile (PAP) for strains Mu208, Δ*cyaA*, Δ*crp*, and Δ*glpR*, from Figure 3a, from two independent experiments with 3 biological replicates each. (b-c) Proportion of colonies resistant to fosfomycin in strains Mu208, mutants, and their complement, from two independent experiments with 3 biological replicates each. **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, b: F (1.760, 8.799) = 799.0, c: F (1.208, 9.665) = 293.3. (d) P_{glpT}*mcherry* expression intensity in the Δ*glpR* mutant among cells that survived or were killed by 32 μg/mL fosfomycin, from three independent experiments; data were determined from 700-1000 cells for each experiment. Data are presented as mean values +/- SD. (e-f) PAP of Mu208 following growth in in M9+0.8% glycerol broth containing 0 or 5 mM cAMP. From two independent experiments with 3 biological replicates each. Data are presented as mean values +/- SD. (f) is an alternative presentation showing each replicate. (g) Proportion of colonies resistant to fosfomycin in strains Mu208, Δ*cyaA* mutant, and the complement, from two independent experiments with 3 biological replicates each. * indicates p= 0.0127 and **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, d: F (1.411, 7.055) = 55.80 (g) Proportion of colonies resistant to fosfomycin in strains indicated (EV indicates empty vector integrated into *att* site, from two independent experiments with 3 biological replicates each. ns indicates p=0.1419 or 0.9936(left to right), *** indicates p= 0.0003 or 0.0001 (left to right) and **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, F (2.748, 13.74) = 319.7. Source data are provided as a Source Data file.



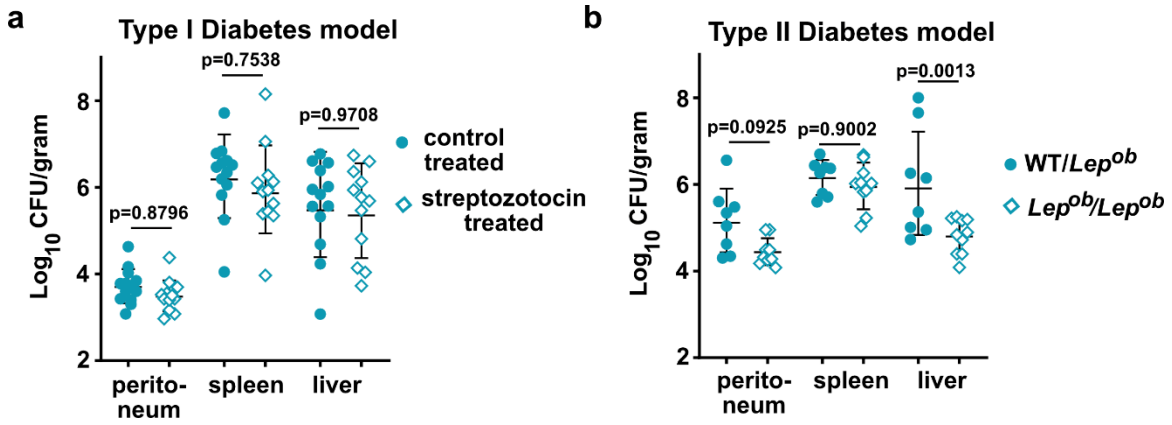
Supplementary Figure 10. Glucose enhances the resistant subpopulation in Mu208. (a-b) PAP of Mu208 and Δ*ptsG* mutant following growth in MHB or MHB+1% glucose, Data are presented as mean values +/- SD. (b) is an alternative presentation of panel a; (a-b) are from three independent experiments with 3 biological replicates each. (c-d) PAP of Mu208 following growth in M9+0.4% glycerol as the sole carbon source or glycerol and glucose as indicated. (c) Data are presented as mean values +/- SD. (d) is an alternative presentation of panel c. (c-d) are from three independent experiments with 3 biological replicates each. (e-f) Alternative presentation of population analysis profiles from strains Mu208, Δ*glpT*, Δ*gshA*, and Δ*fosA*. (e) following growth in M9+ glycerol as the sole carbon source or (f) following growth in M9+glucose as the sole carbon source, from two independent experiments with 3 biological replicates each. Source data are provided as a Source Data file.



Supplementary Figure 11. Glucose enhances the resistant subpopulation in additional carbapenem-resistant *Enterobacter* clinical isolates. Proportion of colonies resistant to fosfomycin following growth in (a) Mueller Hinton broth ±1% glucose and (b) M9 media with glycerol or glucose as the sole carbon sources. (a-b) from two independent experiments with 3 biological replicates each. ns indicates p=0.0909, * indicates p=0.0173, *** indicates p=0.0003 and **** indicates p<0.0001 by one-way ANOVA with Sidak's correction for multiple comparisons, F (9, 50) = 87.55 for a and F (9, 50) = 302.2 for b. Source data are provided as a Source Data file.



Supplementary Figure 12. Glucose enhances the frequency of the fosfomycin resistant subpopulation in *Escherichia* and *Klebsiella* but not *S. aureus*. (a-b, d-e) *Escherichia* or *Klebsiella* isolates were grown in M9 media with 0.8% glycerol or 0.4% glucose as the sole carbon source and (d-e) PAP was performed. The proportion of colonies resistant to (a) 64 µg/mL fosfomycin or (b) 256 µg/mL fosfomycin are presented from the same experiments. (c,f) *S. aureus* isolates were grown in Mueller Hinton broth ±2% glucose and (f) PAP was performed. (c) The proportion of colonies resistant to 32 µg/mL fosfomycin is presented. (a-f) are from three independent experiments with 2 biological replicates each. In (a-c), p values are indicated, by one-way ANOVA with Sidak's correction for multiple comparisons. For (a), F (15, 80) = 22.69, for (b) F (15, 80) = 14.97, for (c) F (9, 50) = 15.50. (d-f) Data are presented as mean values +/- SD. Source data are provided as a Source Data file.



Supplementary Figure 13. Total bacterial burdens in murine models of diabetes. Total colony forming units isolates from indicated tissue following infection with Mu208. Data are presented as geometric mean values +/- geometric SD. Mice were infected with 1.5-2x10^8 colony forming units for ~24 h. (a) Prior to infection, mice were treated with control or streptozotocin (STZ), from three independent experiments with 13 mice total for control and 12 mice total for STZ treated. ns indicates p>0.05 by one-way ANOVA with Šídák's multiple comparisons test, F (5, 69) = 24.14. (b) B6.Cg-*Lep*^{ob}/J heterozygous or homozygous (*Lep*^{ob}/*Lep*^{ob}) mice were infected, from two independent experiments with 8 mice per total for WT/*Lep*^{ob} and 10 mice total (one data point for peritoneum missing) for *Lep*^{ob}/*Lep*^{ob}. p values are displayed, from one-way ANOVA with Šídák's multiple comparisons test, F (5, 47) = 10.30. Source data are provided as a Source Data file.

Supplementary Table 1: Strains and Plasmids		
Strain	**Description**	**Source**
Mu208	*Enterobacter kobei* clinical isolate	GaEIP
Mu819	*Enterobacter cloacae* complex clinical isolate	GaEIP
Δ*recA (ACM9IL_18290)*	In-frame unmarked deletion of *recA*	This study
Δ*fosA (ACM9IL_02510)*	In-frame unmarked deletion of *fosA*	Band and Hufnagel *et al*[2]
Δ*gshA (ACM9IL_18235)*	In-frame unmarked deletion of *gshA*	This study
glpD (*ACM9IL_21870)*	Δ*glpD::kan_FRT* mutation of *glpD*	This study
Mu208 *att*::	pCD13PSK integrated at *attB* site in chromosome of wildtype Mu208	This study
Δ*glpT (ACM9IL_16015)*	In-frame unmarked deletion of *uhpT*	This study
Δ*glpT att*::empty vector	pCD13PSK integrated at *attB* site in chromosome of Mu208 Δ*glpT*	This study
Δ*glpT att*::P_{glpT}*glpT*	pCD13PSK P_{glpT}*glpT* integrated at *attB* site in chromosome of Mu208 Δ*glpT*	This study
Δ*glpT att*::P_{tet}*glpT*	pCD13PSK P_{tet}*glpT* integrated at *attB* site in chromosome of Mu208 Δ*glpT*	This study
Δ*uhpT (ACM9IL_00195)*	In-frame unmarked deletion of *uhpT*	This study
Mu208 *att*::P_{glpT}*mCherry*	pCD13PSK P_{glpT}*mCherry* integrated at *attB* site in chromosome of wildtype Mu208	This study
Mu208 *att*::P_{gshA}*mCherry*	pCD13PSK P_{gshA}*mCherry* integrated at *attB* site in chromosome of wildtype Mu208	This study
Δ*fosA att*::P_{glpT}*mCherry*	pCD13PSK P_{glpT}*mCherry* integrated at *attB* site in chromosome of Mu208 Δ*fosA*	This study
Δ*crp (ACM9IL_21625)*	In-frame unmarked deletion of *crp*	This study
Δ*glpR (ACM9IL_21855)*	In-frame unmarked deletion of *glpR*	This study
Δ*cyaA (ACM9IL_22585)*	In-frame unmarked deletion of *cyaA*	This study
Δ*glpT* Δ*crp*	In-frame unmarked deletion of *crp* and *glpT*	This study
Δ*glpT* Δ*crp att*::empty vector	pCD13PSK integrated at *attB* site in chromosome of Mu208 Δ*glpT* Δ*crp*	This study
Δ*glpT* Δ*crp att*::P_{glpT}*glpT*	pCD13PSK P_{glpT}*glpT* integrated at *attB* site in chromosome of Mu208 Δ*glpT* Δ*crp*	This study
Δ*glpT* Δ*crp att*::P_{tet}*glpT*	pCD13PSK P_{tet}*glpT* integrated at *attB* site in chromosome of Mu208 Δ*glpT* Δ*crp*	This study
Δ*glpR att*::P_{glpT}*mCherry*	pCD13PSK P_{glpT}*mCherry* integrated at *attB* site in chromosome of Mu208 Δ*glpR*	This study
Δ*crp att*::P_{glpT}*mCherry*	pCD13PSK P_{glpT}*mCherry* integrated at *attB* site in chromosome of Mu208 Δ*crp*	This study
Mu208 *att*::P_{crp}*mCherry*	pCD13PSK P_{crp}*mCherry* integrated at *attB* site in chromosome of wildtype Mu208	This study
Δ*ptsG (ACM9IL_08775)*	In-frame unmarked deletion of *ptsG*	This study
Mu898, Mu909, Mu866, Mu1197	*Enterobacter cloacae* clinical isolates	GaEIP
Mu1004, Mu499, Mu582, Mu597, Mu665, Mu772	*Escherichia coli* clinical isolates	GaEIP
Mu1331, Mu1407	*Escherichia albertii* clinical isolates	GaEIP
Mu234, Mu979, Mu1343, Mu1516, Mu1010, Mu1279, Mu669, Mu754	*Klebsiella* clinical isolates	GaEIP
JE2 and Newman	*S. aureus* laboratory isolates	Brian Conlon lab
BC780, BC784, BC814	*S. aureus* cystic fibrosis clinical isolate	Lu et al[3]
Plasmids		
Name	**Description**	**Source**
pFD1	Plasmid encoding an IPTG inducible *Himar1* mariner transposase, pi-dependent origin, carbenicillin selection	Rubin *et al*[4]
pEXR6K_FRT_*Kan*	Encodes FRT_*kan*, template for PCR, a derivative of pEXT100 but with R6K origin that is pi-dependent	This study
pKD46-tet	Encodes lambda red recombinase, tetracycline resistant derivative of pKD46, temperature sensitive origin	Datsenko and Wanner[5]
pCP20	Encodes Flp recombinase, temperature sensitive origin	Cherepanov and Wackernagel[6]
pINT-ts-tet	Encodes helper machinery for pCD13PSK, temperature sensitive origin	Haldimann and Wanner[7]
pCD13PSK	Pi-dependent origin plasmid with homology to *attB* site	Haldimann and Wanner[7]
pBAV1K-T5-gfp	Multi-species vector used for complementation	Bryksin and Matsumura[8]
pBAV	pBAV1K-T5-*gfp* with *gfp* excised by Hifi assembly	This study

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