Supplementary Figures

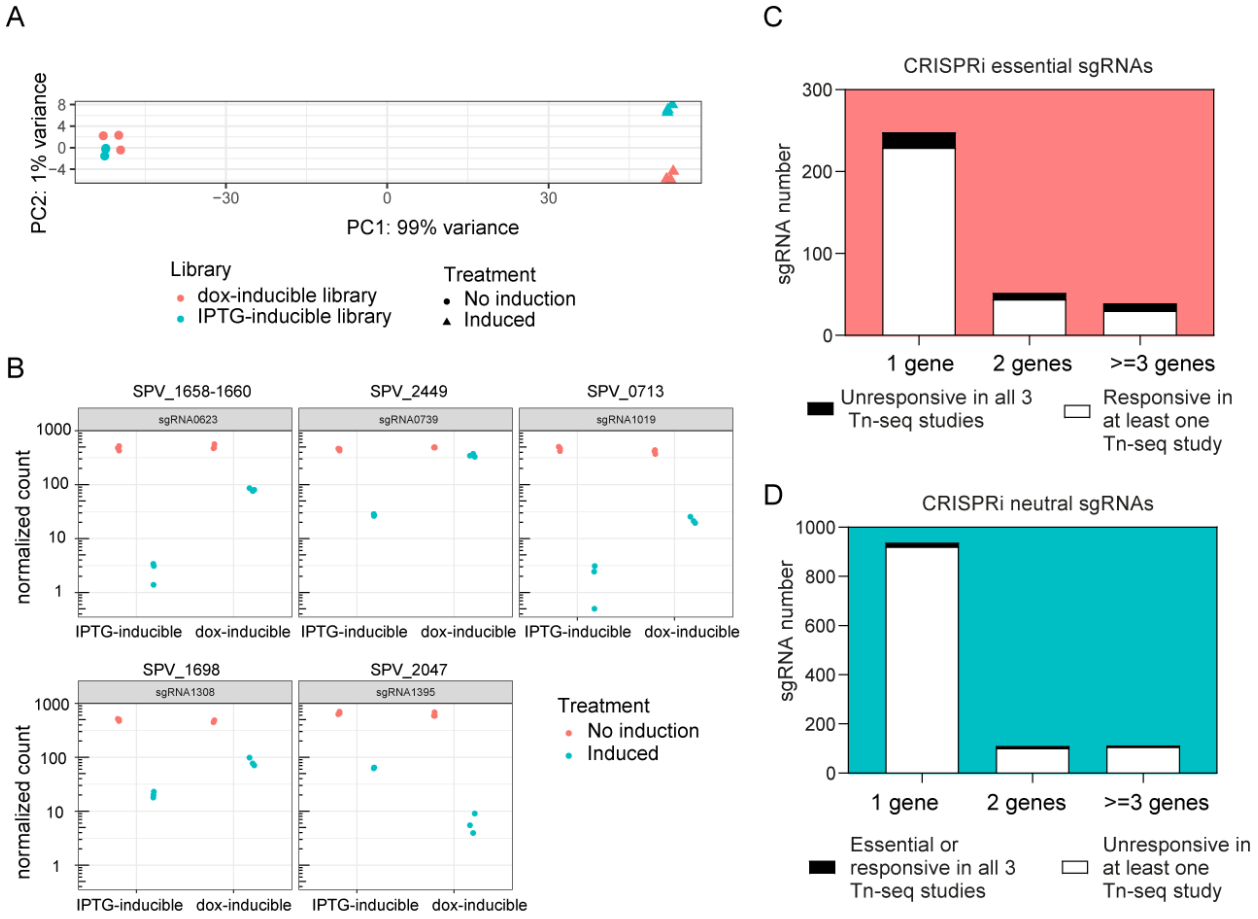

Figure S1. CRISPRi-seq with the IPTG- and dox-inducible libraries in C+Y medium. (A) PCA analysis of the samples grown in C+Y medium with or without inducer. (B) The sgRNAs that showed significantly different fold change of control sample and induced samples between the dox-inducible library and IPTG-inducible library. (C-D) Comparison of the essential gene list identified by CRISPRi-seq and Tn-seq. Related to Figure 3.

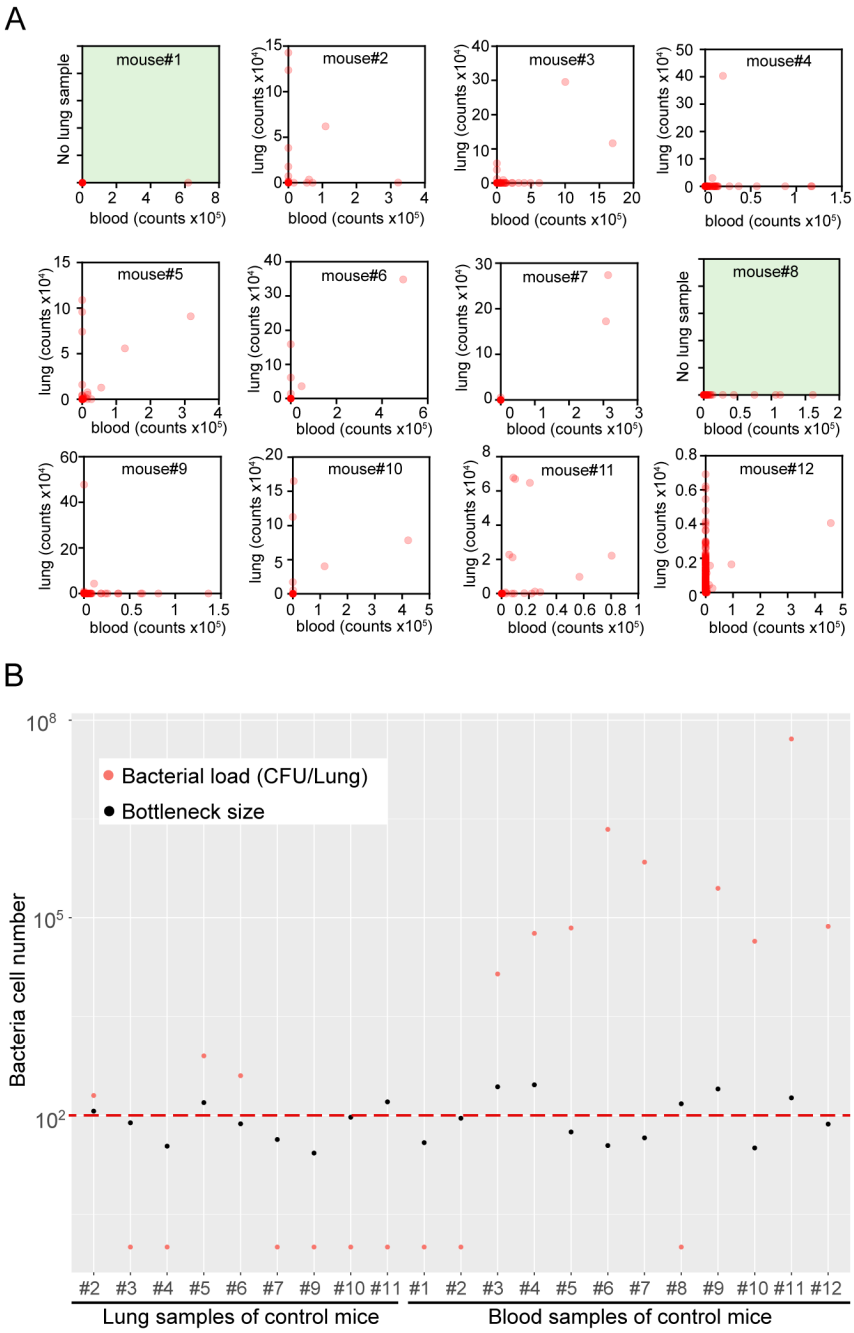

Figure S2. Heterogeneity of infection in murine pneumonia model at 48 hpi. (A) Correlation of sgRNA abundance between lung and blood samples per mouse at 48 hpi, in the control group (not treated with doxycycline). Note for mouse #3 and mouse #8, we failed to collect bacteria from lung samples, so only the sgRNA abundance of blood samples was shown for these two mice. (B) Comparison of bacterial load to estimated bottleneck size in the mouse pneumonia model at 48 hpi. Bacterial load (CFU/Lung) was determined by plating homogenized tissue and numerating colony numbers. Notice that 100 CFU/Lung is the detective limits (red dash line). The red dots below 100 represents bacterial load under detective limits. Bottleneck size was estimated on the basis of allele (here: sgRNA) frequencies in the pool before and after infection as described in the materials and methods. Related to Figure 4.

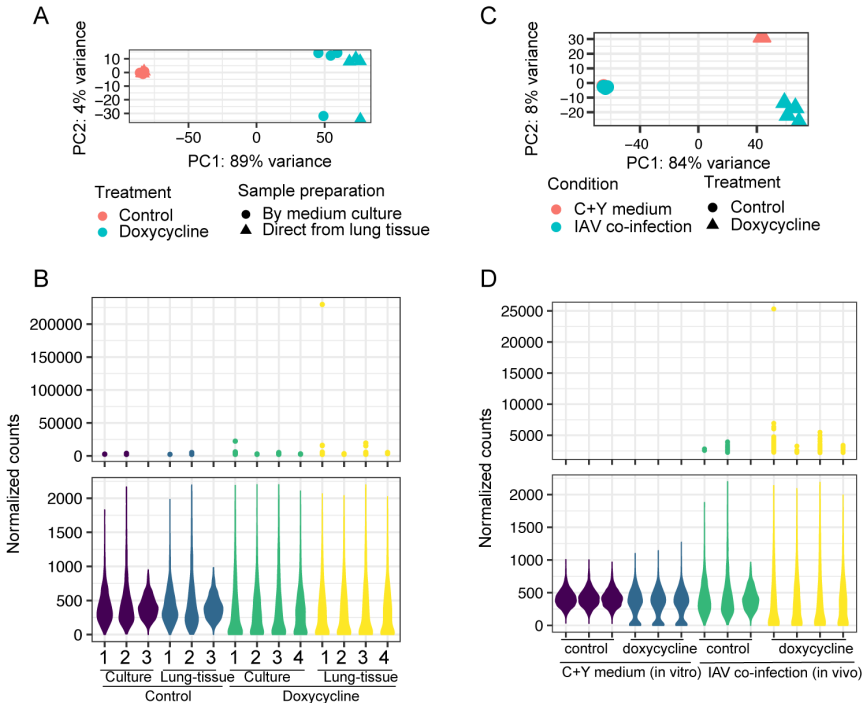

Figure S3. CRISPRi-seq in IAV superinfection model with dox-inducible library. (A) PCA analysis of the samples collected by culturing bacteria from lung tissue in THY medium, or directly from lung tissue without further culturing, with or without inducer. (B) Distribution of normalized counts of sgRNAs in the samples as in panel A. (C) PCA analysis of the *in vitro* (C+Y medium) and *in vivo* (IAV co-infection) samples, with or without induction. (D) Distribution of normalized counts of sgRNAs in the samples as in panel C. Related to Figure 6 and STAR methods.

Table S8. Strains and plasmids used in this study, related to STAR methods

Strains/Plasmids	Genotype	Reference
S. pneumoniae		
D39V	Serotype 2 strain, wild-type	(Slager et al., 2018)
DCI23	D39V, Δ*bgaA*::*P_{lac}-*dcas9sp* (tet^R); Δ*prs1*::PF6-*lacI* (Gm^R)	(Liu et al., 2017)
VL1780	D39V, *hlpA*::*hlpA_hlpA-mScarlet-I* (cam^R)	(Kurushima et al., 2020)
XL28	D39V, Δ*bgaA*::*P_{lac}-*dcas9sp* (tet^R); Δ*prs1*::PF6-*lacI* (Gm^R); **cil*::P3-*luc* (kan^R), Δ*CEP*::P3-*sgRNAluc* ($spec^R$)	(Liu et al., 2017)
D-T-PEP9Ptet	D39V, Δ*prs1*::PF6-*tetR* (Gm^R); Δ*CEP*::P_{tet}-*luc-gfp* ($spec^R$)	(Sorg et al., 2019)
VL2210	D39, Δ*prs1*::PF6-*tetR*	This study
VL2212	D39, Δ*prs1*::PF6-*tetR*, Δ*bgaA*:: *P_{tet}-*dcas9*	This study
VL2339	D39, Δ*prs1*::PF6-*tetR*, Δ*bgaA*:: *P_{tet}-*dcas9*, *cil**::P_{lac}-*mNeonGreen* (Kan^R)	This study
VL2351	D39, Δ*prs1*::PF6-*tetR*, Δ*bgaA*::P_{tet}-*dcas9*, *cil**::P_{lac}-*mNeonGreen* (Kan^R), *hlpA*::*hlpA-mScarlet-I* (cam^R)	This study
VL3106	D39V, Δ*ccnC*::eryR	This study
VL3107	D39V, Δ*purA*::eryR	This study
VL3108	D39V, Δ*srf-28*::eryR	This study
VL3109	D39V, Δ*pezT*::eryR	This study
VL3110	D39V, Δ*ylmE*::eryR	This study
VL3111	D39V, ΔSPV_0007::eryR	This study
VL3112	D39V, ΔSPV_1234::eryR	This study
VL3113	D39V, ΔSPV_1235::eryR	This study
VL3114	D39V, Δ*pezA-T*::eryR	This study
VL3168	D39V, Δ*ccnC*::eryR, *ZIP**::P-*ccnC* (Native promoter of *purA-ccnC* operon was used)	This study
VL3169	D39V, Δ*purA*::eryR, *ZIP**::P-*purA* (Native promoter of *purA-ccnC* operon was used)	This study
VL3462	D39V, Δ*metK*::eryR	This study
VL3508	D39V, Δ*cps*::eryR	This study
VL237	D39V, Δ*ply*::cmR	(Hassane et al., 2017)
Plasmids		
pPEPZ-sgRNAclone	*ZIP**', specR, P3, mCherry, sgRNA(dCas9handling+terminator), '*ZIP**	This study
pPEP8T4-1	*CEP**', $spec^R$, *tetR*, PT4-1, *luc-gfp*, '*CEP**	(Sorg et al., 2019)
pASR110 (pPEPZ-P_{lac}-mNeonGreen)	*ZIP**', $spec^R$, P_{lac}-mNeonGreen, '*ZIP**	(Keller et al., 2019)
pPEPY-P_{lac}	*cil**', kan^R, P_{lac}, MCS, '*cil**	(Keller et al., 2019)
pJWV502	*ampR, bgaA', tet^R,* P_{Zn}-*gfp, ery^R, 'bgaA*	(Liu et al., 2017)

Notes:

1. cam^R: chloramphenicol resistance; ery^R: erythromycin resistance; Gm^R: gentamycin resistance; kan^R: kanamycin resistance; $spec^R$: spectinomycin resistance; tet^R: tetracycline resistance
2. *P_{lac}: The IPTG inducible promoter

3. *P_{tet}: the tetracycline inducible promoter. The here used P_{tet} represents PT4-1in our previous study (Sorg et al., 2019)
4. *cil: chromosome intergration locus, represents a non-coding region between SPD_0422 and SPD_0423
5. *ZIP: pPEPZ intergration site at locus *spv_2417* (Keller et al., 2019)