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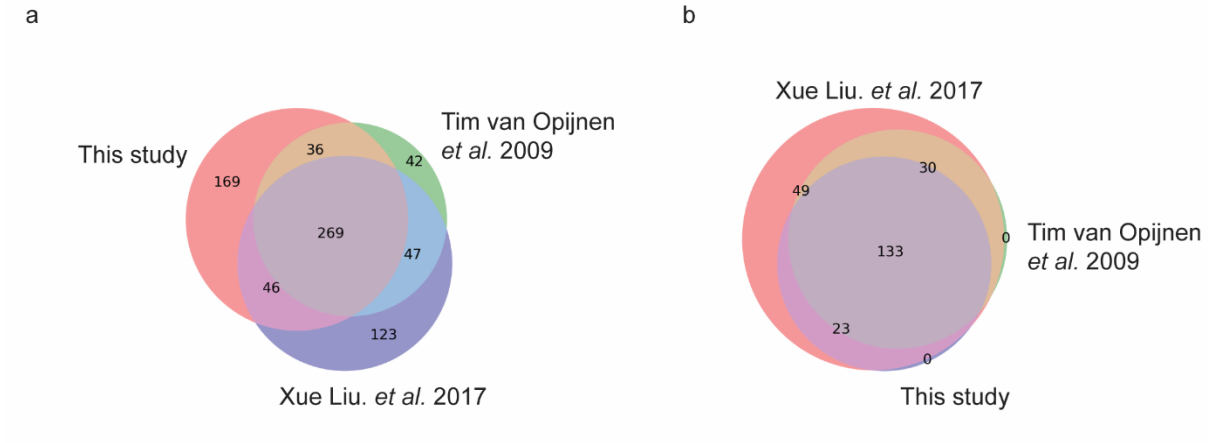
Supplementary information

CRISPRi-seq for genome-wide fitness quantification in bacteria

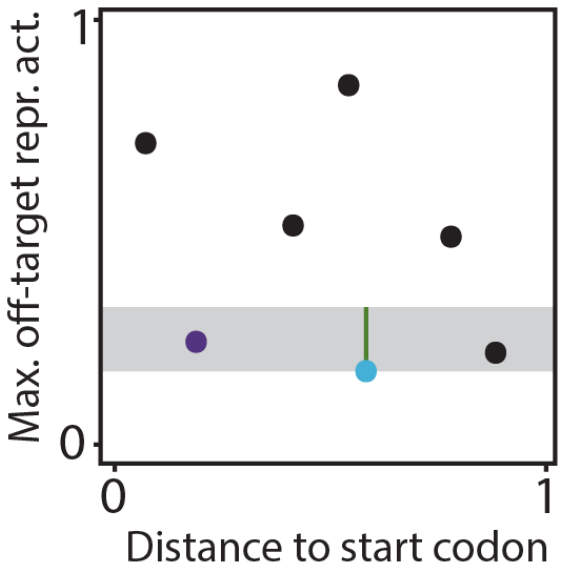
In the format provided by the authors and unedited

Supplementary Information

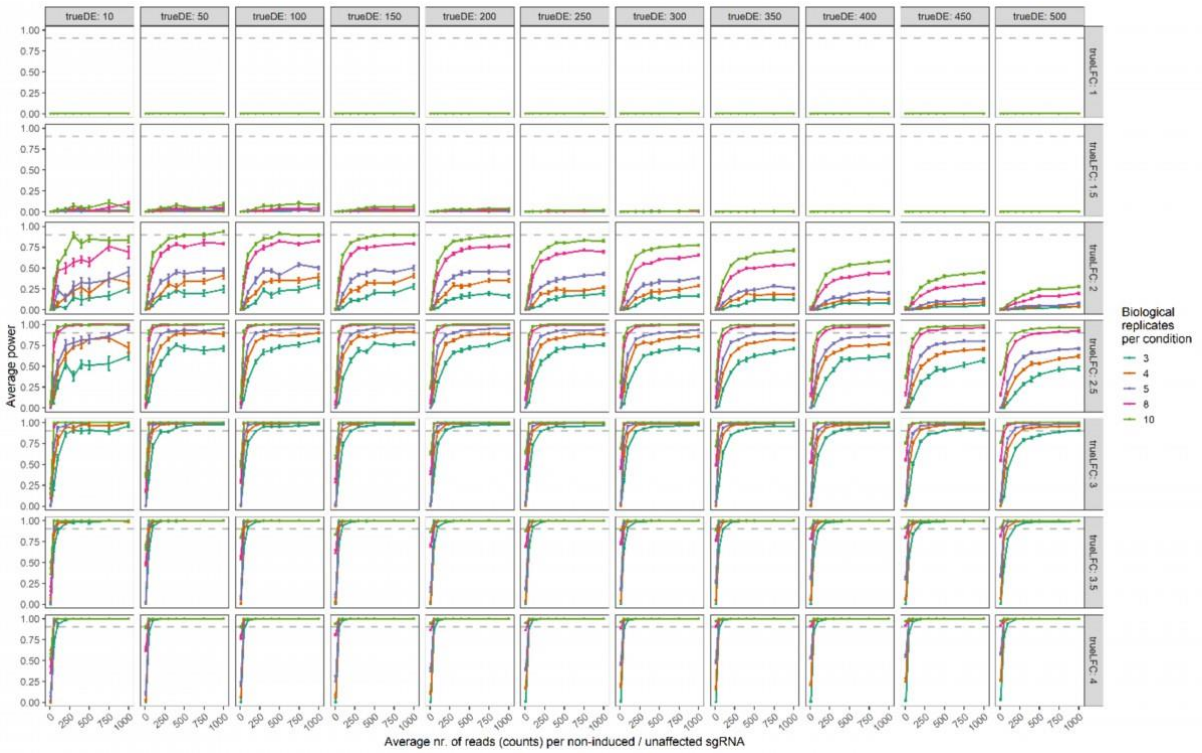
Supplementary Figures



Supplementary Fig. 1
Comparison of Tn-seq and CRISPRi-seq fitness screening results
Venn diagrams of locus essentiality comparisons between two independent Tn-seq studies performed in *S. pneumoniae* D39 (Liu, X. *et al.*, *Mol. Syst. Biol.* **13**, 931, 2017; Van Opijnen, T., *et al.*, *Nat. Methods* **6**, 767–772, 2009), and the current CRISPRi-seq study (Liu, X. *et al.*, *Cell Host Microbe* **29**, 107-120.e6, 2021). Comparison of locus essentiality overlap when considering **a,** individual genes; **b,** whole operons. An operon was considered essential if at least one gene of the same operon was deemed essential. Regarding CRISPRi-seq, all the genes of any given operon were considered essential if the operon was deemed essential. Only loci marked as essential in liquid C+Y medium were considered for comparison for CRISPRi-seq. The medium differed from the one used by Van Opijnen *et al.* (Todd Hewitt broth supplemented with yeast extract).



Supplementary Fig. 2
Schematic representation of optimal sgRNA selection
All zero-mismatch candidate sgRNAs are considered per annotated feature, here depicted as points for an hypothetical feature with seven candidates that can target it. First, for each candidate the mismatch-based relative expected repression activity of all its binding sites outside the currently considered feature are screened, and the maximum is registered (y-axis). The minimum of the thus found maximum off-target repression activities of all candidates is determined (blue point) and all candidates within a user-defined range (green line; default: 0.4) of that minimum survive the first selection round. Second, of the remaining candidates (those in the grey area), the one with the minimum distance to the feature start (x-axis) is selected as optimal candidate (purple point) for that feature.



Supplementary Fig. 3

Naïve power simulations of differential sgRNA depletion calling by DESeq2

Simulation study to explore the influence of the number of replicate samples, number of differentially depleted sgRNAs, depletion effect sizes and number of reads per sgRNA on statistical power (the probability of detecting truly differentially depleted sgRNAs with DESeq2). For every combination of number of truly differentially depleted sgRNAs (trueDE) between two conditions and the corresponding true $\log_2$ fold change (trueLFC), a sample of 1,499 sgRNAs was drawn for the specified number of replicate samples from a negative binomial distribution with a mean of either 10, 50, 100, 200, 300, 400, 500, 750 or 1,000 for control and truly non-depleted sgRNAs, as displayed on the x-axis. The dispersion parameter for every draw was estimated as $\frac{4}{mean} + 0.1$. Mean read counts for truly depleted sgRNAs were computed as the trueLFC of the control mean read counts, as on the x-axis, which were then used to simulate the depleted sgRNA count samples in the same fashion. Power was calculated as the fraction of sgRNAs that was correctly called as significantly depleted by DESeq2 (Love, M. I., *et al.*, *Genome Biol.* **15**, 550, 2014), testing with an alpha of 0.05 and an lfcThreshold of 1. For every combination, this process was repeated ten times, of which averages and SEM are plotted in the figure. Horizontal, dotted grey line represents an average power of 0.9. Note that this is a very naïve simulation and should be taken with a grain of salt, as for every simulation round, every sgRNA can have only either of two average read counts and their dispersion is assumed to be related according to the specified formula. In addition to these naïve assumptions, the shown dynamics may very well be different for different sgRNA library sizes, organisms and sequencing methods. For large trueDE, artefacts of the read count normalization start to artificially affect the outcomes due to inflation of the total read count per sample; this is an artefact of the naïve setup of the analysis. For larger trueLFC, the calls appear to be mostly correct, stressing the importance of pooled growth for a sufficient number of generations in assessing essentiality to achieve proper sgRNA selection.

Supplementary Methods

Protocol for C+Y medium

Materials

- L-Cysteine HCl (Sigma-Aldrich, cat. no. C7880-500MG)
- Sodium acetate (Sigma-Aldrich, cat. no. S5636-1KG)
- Casein hydrolysate (OXOID, cat. no. LP0041)
- L-Tryptophan (Sigma-Aldrich, cat. no. T8941-25G)
- Potassium phosphate dibasic (K_2HPO_4) (Sigma-Aldrich, cat. no. 60356-1KG)
- L-Glutamine (Sigma-Aldrich, cat. no. G3126-100G)
- Sodium pyruvate (Sigma-Aldrich, cat. no. P2256-25G)
- Bovine Serum Albumin (BSA) (Sigma-Aldrich, cat. no. A9647-50G)
- Uridine (Sigma-Aldrich cat. no. U6381-5G)
- Adenosine (Sigma-Aldrich cat. no. 01890-25G)
- Sucrose (PanReac AppliChem cat. no. A3935-5KG)
- Glucose (PanReac Applichem, cat. no. A1422-1KG)
- Yeast Extract (BD Difco™ cat. no. 211929)
- Hydrochloric acid (HCl) (Sigma-Aldrich, cat. no. 320331-500ML)
- Biotin (Sigma-Aldrich, cat. no. B4639)
- Nicotinic acid (Sigma-Aldrich, cat. no. 72309-100G)
- Pyridoxine hydrochloride (Sigma-Aldrich, cat. no. P9755-25G)
- Calcium pantothenate (Sigma-Aldrich, cat. no. C8731-25G)
- Thiamine hydrochloride (Sigma-Aldrich, cat. no. T4625-10G)
- Riboflavin (Sigma-Aldrich, cat. no. R9504-25G)
- $FeSO_4.7H_2O$ (PanReac Applichem, cat. no. A1035-500G)
- $CuSO_4.5H_2O$ (Sigma-Aldrich, cat. no. 61240-250G)
- $ZnSO_4.7H_2O$ (Sigma-Aldrich, cat. no. Z0251-100G)
- $MnCl_2.4H_2O$ (Sigma-Aldrich, cat. no. 1059270100-100G)
- L-Asparagine anhydrous (Sigma-Aldrich, cat. no. A0884-25G)
- Choline chloride (Sigma-Aldrich, cat. no. C7017-5G)
- $CaCl_2$ anhydrous (Sigma-Aldrich, cat. no. 746495-1KG)
- $MgCl_2.6H_2O$ (Sigma-Aldrich, M2670-500G)
- Milli-Q (MQ) water

Procedure

1. Prepare Pre-C according to the following table:

Component	**To make 4 L of Pre-C**	**To make 2 L of Pre-C**	**To make 1 L of Pre-C**
L-Cysteine HCl	48.28 mg	24.014 mg	12.07 mg
Sodium acetate	8.4 g	4.2 g	2.1 g
Casein hydrolysate	21.48 g	10.74 g	5.37 g
L-Tryptophane	25.7 mg	12.8 mg	6.4 mg
K2HPO4	36.4 g	18.2 g	9.1 g
MQ water	4 L	2 L	1 L

   Stir it for at least 15 minutes to dissolve all the components properly. Sterilize the solution by autoclavation at 121ºC for 20 min. The autoclaved Pre-C can be kept at room temperature avoiding light exposure for months.

2. Preparation of the components.

Components	**Protocol**
2.19% Glutamine	Weigh 1.095 g of glutamine and add into 50 ml MQ water. Stir to dissolve and sterilize by filtration with 0.22 μm filter. Aliquot in 1.5-ml Eppendorf tubes and keep at -20ºC for months.

1.2% Na pyruvate	Weigh 6 g of Na pyruvate and add into 500 ml MQ water. Stir to dissolve and sterilize by filtration with 0.22 μm filter. Aliquot into 50-ml Falcon tubes. Keep at 4ºC for months.
3.2% BSA	Weigh 16 g of BSA and add into 500 ml MQ water. Stir to dissolve and sterilize by filtration with 0.22 μm filter. Aliquot in 50-ml Falcon tubes. Keep at 4ºC for months.
Mn Cl_2 0,4 mM	Weigh 7.9 mg of $MnCl_2.4H_2O$ and add into 100 ml MQ Water. Stir to dissolve and sterilize by filtration with 0.22 μm filter. Aliquot into 15-ml Falcon tubes. Keep at 4ºC for months.
Uridine/Adenosine	Weigh 400 mg of uridine and 400 mg of adenosine, and add into 500 ml MQ water. This results in 0.8 mg/ml Uridine plus 0.8 mg/ml Adenosine in MQ water. Stir to dissolve and sterilize by filtration with 0.22 μm filter. Aliquot in 50-ml Falcon tubes. Keep at 4ºC for months.
32.32% Sucrose	Weigh 64,64g of sucrose and transfer into a 200-ml bottle. Add water to final volume of the solution as 200 ml. Stir to dissolve and sterilize by filtration with 0.22 μm filter. Aliquot into 15-ml Falcon tubes. Keep at 4ºC for months.
8% Glucose	Weigh 40 g of glucose and add into 500 ml MQ water. Stir to dissolve and sterilize by filtration with 0.22 μm filter. Aliquot in 50-ml Falcon tubes. Keep at 4ºC for months.
10% Yeast Extract	Weigh 50 g of yeast extract and add into 500 ml MQ water. Stir to dissolve completely and sterilize by autoclaving. Aliquot in 50-ml Falcon tubes. Keep at 4ºC for months.
1N HCl	Add 41.5 ml of 37% HCl solution to 400 ml MQ water slowly. Adjust the volume to 500 ml with MQ water. Sterilize by filtration with 0.22 μm filter. Aliquot in 50-ml Falcon tubes. Keep at 4ºC for months.
ADAMS III	See the protocols below

ADAMS I and ADAMS II are required to prepare ADAMS III. So, first prepare ADAMS I and ADAMS II.

Prepare ADAMS I:

Components	**Amount**
Biotin	75 mg
Nicotinic acid	75 mg
Pyridoxine hydrochloride	87.5 mg
Ca pantothenate	300 mg
Thiamine hydrochloride	80 mg
Riboflavin	35 mg
MQ water	500 ml

Mix the solution well and adjust the pH to 7.0 with 1N NaOH (which needs ~1 ml of 1 N NaOH)

Sterilize the solution by incubating in boiling water for 15 min. Keep at 4ºC for months and avoid light exposure.

Prepare ADAMS II (10×)

Components	**Amount**
$FeSO_4.7H_2O$	500 mg
$CuSO_4.5H_2O$	500 mg
$ZnSO_4.7H_2O$	500 mg
$MnCl_2.4H_2O$	200 mg
MQ water	90 ml
HCl 37%	10 ml

Mix the solution well and sterilize by incubating in boiling water for 15 min. Keep at 4ºC for months and avoid light exposure.

ADAMS III

Components	**Amount**
ADAMS I	128 ml
ADAMS II (10×**)**	3.2 ml
L-Asparagine anhydrous	1.4 g
Choline chloride	160 mg
$CaCl_2$ anhydrous	0.4 g
$MgCl_2.6H_2O$	16 g
MQ water	650 ml

Mix the solution assembled above well, and then adjust the pH to 7.6 with about 540 µl of 10 N NaOH. Bring the final volume to 800 ml with MQ water. Sterilize the solution with 0.22 µM filter, and keep at 4ºC for months.

CRITICAL STEP Please note that it is tricky to make ADAMS III, because addition of 10 N NaOH to adjust the pH leads to precipitation and the pH cannot be accurately measured with the precipitation in the solution. So, we suggest to add 540 µl of 10 N NaOH into the solution, and then put the solution on magnetic stirrer to let the precipitation re-dissolve, which takes 1-2 hours. Once the precipitation is dissolved, continue the pH adjustment. After pH adjustment, put the solution back to stirrer for another 10 min before filtering. After long-term storage, precipitation will form in the solution. Shake the bottle to dissolve the precipitation before usage.

3. Make acid C+Y medium. Assemble the components according to the table below to make the acid C+Y medium. Handle all the compenents under a flow hood to avoid contamination.

Components	**Amount**
Pre-C	40 ml
2.19% Glutamine	40 µl
1.2% Na pyruvate	1 ml
3.2% BSA	1 ml
$MnCl_2$ 0.4 mM	40 µl
Uridine/Adenosine	1 ml
32.32% Sucrose	40 µl
8% Glucose	1 ml
10% Yeast Extract	1 ml
1N HCl	1 ml
ADAMS III	1 ml

We recommend to assemble the acid C+Y medium freshly. The assembled acid C+Y medium can be kept at 4ºC for 3 days.