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High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*

Xue Liu1,2, Clement Gallay3, Morten Kjos1,3, Arnau Domenech1, Jelle Slager1, Sebastiaan P van Kessel1, Kevin Knoops4, Robin A Sorg1, Jing-Ren Zhang2,5 & Jan-Willem Veening1,5,6

Abstract

Genome-wide screens have discovered a large set of essential genes in the opportunistic human pathogen *Streptococcus pneumoniae*. However, the functions of many essential genes are still unknown, hampering vaccine development and drug discovery. Based on results from transposon sequencing (Tn-seq), we refined the list of essential genes in *S. pneumoniae* serotype 2 strain D39. Next, we created a knockdown library targeting 348 potentially essential genes by CRISPR interference (CRISPRi) and show a growth phenotype for 254 of them (73%). Using high-content microscopy screening, we searched for essential genes of unknown function with clear phenotypes in cell morphology upon CRISPRi-based depletion. We show that SPD_1416 and SPD_1417 (renamed to MurT and GatD, respectively) are essential for competence, and that SPD_1198 and SPD_1197 (renamed to TarP and TarQ, respectively) are responsible for the polymerization of teichoic acid (TA) precursors. This knowledge enabled us to reconstruct the unique pneumococcal TA biosynthetic pathway. CRISPRi was also employed to unravel the role of the essential Clp-proteolytic system in regulation of competence development, and we show that ClpX is the essential ATPase responsible for ClpP-dependent repression of competence. The CRISPRi library provides a valuable tool for characterization of pneumococcal genes and pathways and revealed several promising antibiotic targets.

Keywords: bacterial cell wall; competence; DNA replication; gene essentiality; teichoic acid biosynthesis

Subject Categories: Chromatin, Epigenetics, Genomics & Functional Genomics; Genome-Scale & Integrative Biology; Microbiology, Virology & Host Pathogen Interaction

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Introduction

*Streptococcus pneumoniae* (pneumococcus) is a major cause of community-acquired pneumonia, meningitis, and acute otitis media and, despite the introduction of several vaccines, remains one of the leading bacterial causes of mortality worldwide (Prina et al., 2015). The main antibiotics used to treat pneumococcal infections belong to the beta-lactam class, such as amino-penicillins (amoxicillin, ampicillin) and cephalosporines (cefotaxime). These antibiotics target the penicillin binding proteins (PBPs), which are responsible for the synthesis of peptidoglycan (PG) that plays a role in the maintenance of cell integrity, cell division, and anchoring of surface proteins (Sham et al., 2012; Kocaoglu et al., 2015). The pneumococcal cell wall furthermore consists of teichoic acids (TA), which are anionic glycopolymers that are either anchored to the membrane (lipo TA) or covalently attached to PG (wall TA) and are essential for maintaining cell shape (Brown et al., 2013; Massidda et al., 2013). Unfortunately, resistance to most beta-lactam antibiotics remains alarmingly high. For example, penicillin non-susceptible pneumococcal strains colonizing the nasopharynx of children remain above 40% in the United States (Kaur et al., 2016), despite the effect of the pneumococcal conjugate vaccines. Furthermore, multidrug resistance in *S. pneumoniae* is prevalent and antibiotic resistance determinants and virulence factors can readily transfer between strains via competence-dependent horizontal gene transfer (Chewapreecha et al., 2014; Johnston et al., 2014; Kim et al., 2016). For these reasons, it is crucial to understand how competence is regulated and to identify and characterize new essential genes and pathways. Interestingly, not all proteins within the pneumococcal PG and TA biosynthesis pathways are known (Massidda et al., 2013), leaving room for discovery of new potential antibiotic targets. For instance, not all enzymes in the biosynthetic route to lipid II, the precursor of PG, are known and annotated in *S. pneumoniae*. The pneumococcal TA biosynthetic pathway is even more enigmatic, and it is unknown which genes code for the enzymes responsible for polymerizing TA precursors (Denapaite et al., 2012).

Several studies using targeted gene knockout and depletion/overexpression techniques as well as transposon sequencing (Tn-seq)
have aimed to identify the core pneumococcal genome (Thanassi et al., 2002; Song et al., 2005; van Opijnen et al., 2009; van Opijnen & Camilli, 2012; Zomer et al., 2012; Mobegi et al., 2014; Verhagen et al., 2014). These genome-wide studies revealed a core genome of around 400 genes essential for growth either in vitro or in vivo. Most of the essential pneumococcal genes can be assigned to a functional category on the basis of sequence homology or experimental evidence. However, per the most recent gene annotation of the commonly used S. pneumoniae strain D39 (NCBI, CP000410.1, updated on 31-JAN-2015), approximately one-third of the essential genes belong to the category of “function unknown” or “hypothetical” and it is likely that several unknown cell wall synthesis genes, such as the TA polymerase, are present within this category.

To facilitate the high-throughput study of essential genes in S. pneumoniae on a genome-wide scale, we established CRISPRi (clustered regularly interspaced short palindromic repeats interference) for this organism. CRISPRi is based on expression of a nuclease-inactive Streptococcus pyogenes Cas9 (dCas9), which together with expression of a single-guide RNA (sgRNA) targets the gene of interest (Bikard et al., 2013; Qi et al., 2013; Peters et al., 2016). When targeting the non-template strand of a gene by complementary base-pairing of the sgRNA with the target DNA, the dCas9-sgRNA-DNA complex acts as a roadblock for RNA polymerase (RNAP) and thereby represses transcription of the target genes (Qi et al., 2013; Peters et al., 2016) (Fig 1A). Note that S. pneumoniae does not contain an endogenous CRISPR/Cas system, consistent with interference with natural transformation and thereby lateral gene transfer that is crucial for pneumococcal host adaptation (Bikard et al., 2012).

Using Tn-seq and CRISPRi, we refined the list of genes that are either essential for viability or for fitness in S. pneumoniae strain D39 (Avery et al., 1944). To identify new genes involved in pneumococcal cell envelope homeostasis, we screened for essential genes of unknown function (as annotated in NCBI), with a clear morphological defect upon CRISPRi-based depletion. This identified SPD_1416 and SPD_1417 as essential peptidoglycan synthesis proteins (renamed to MurT and GatD, respectively) and SPD_1198 and SPD_1197 as essential proteins responsible for precursor polymerization in TA biosynthesis (hereafter called TarP and TarQ, respectively). Finally, we demonstrate the use of CRISPRi to unravel gene regulatory networks and show that ClpX is the ATPase subunit that acts together with the ClpP protease as a repressor for competence development.

Results

Identification of potentially essential genes in S. pneumoniae strain D39

While several previous studies have identified many pneumococcal genes that are likely to be essential, the precise contribution to pneumococcal biology has remained to be defined for most of these genes. Here, we aim to characterize the functions of these proteins in the commonly used S. pneumoniae serotype 2 strain D39 by the CRISPRi approach. Therefore, we performed Tn-seq on S. pneumoniae D39 grown in C+Y medium at 37°C, our standard laboratory condition (see Materials and Methods). We included all genes that we found to be essential in our Tn-seq study, and added extra genes that were found to be essential by previous Tn-seq studies with a serotype 4 strain TIGR4 (van Opijnen et al., 2009; van Opijnen & Camilli, 2012) in the CRISPRi library (see below). Finally, 391 potentially essential genes were selected, and the genes are listed in Dataset EV1.

CRISPRi enables tunable repression of gene transcription in S. pneumoniae

To develop the CRISPR interference system, we first engineered the commonly used LacI-based isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible system for S. pneumoniae (see Materials and Methods). The dcas9 gene was placed under control of this new IPTG-inducible promoter, named P lac, and was integrated into the chromosome via double crossover (Fig 1A and B). To confirm the reliability of the CRISPRi system, we tested it in a reporter strain expressing firefly luciferase (lac), in which an sgRNA targeting lac was placed under the constitutive P3 promoter (Sorg et al., 2015) and integrated at a non-essential locus (Fig 1B). To obtain high efficiency of transcriptional repression, we used the optimized sgRNA sequence as reported previously (Chen et al., 2013) (Fig EV1A).

Induction of dCas9 with 1 mM IPTG resulted in quick reduction in luciferase activity; ~30-fold repression of luciferase expression was obtained within 2 h without substantial impact on bacterial growth (Fig 1C). Furthermore, the level of repression was tunable by using different concentrations of IPTG (Fig 1C). To test the precision of CRISPRi in S. pneumoniae, we determined the transcriptome of the sgRNA lac strain (strain XL28) by RNA-Seq in the presence or absence of IPTG. The data were analyzed using Rockhopper (McClure et al., 2013) and T-Rex (de Jong et al., 2015). The RNA-Seq data showed that the expression of dCas9 was stringently repressed by LacI without IPTG and was upregulated ~600-fold upon addition of 1 mM IPTG after 2.5 h. Upon dCas9 induction, the lac gene was significantly repressed (~84-fold) (Fig 1D). Our RNA-Seq data showed that the genes (spd_0424, spd_0425, lacE-1, lacC-1, lacF-1) that are downstream of lac, which was driven by a strong constitutive promoter without terminator, were significantly repressed as well (Appendix Fig S1A). This confirms the reported polar effect of CRISPRi (Qi et al., 2013). In addition, induction of dCas9 in the sgRNA-deficient strain XL29 (Fig EV1B) led to no repression of the target gene (Fig EV1C). By comparing strains with or without sgRNA, we found that repression in our CRISPRi system is_stringently dependent on the expression of both dCas9 and the sgRNA, and detected no basal level repression (Fig EV1C). Furthermore, we compared the transcriptome of lac reporter strains with sgRNAlac (strain XL28) and without sgRNAlac (strain XL29) both grown in the presence of 1 mM IPTG. This showed that galT-2, galK, and galR were upregulated in both strains, indicating that these genes are activated in response to the inducer IPTG and not by the CRISPRi system itself (Dataset EV2). We also noted a slight repression of several competence genes in both XL28 and XL29 with 1 mM IPTG (Dataset EV2). Since this repression does not rely on the presence of a functional CRISPRi system, we anticipate that these changes are due to the noisy character of the competence system (Aprianto et al., 2016; Prudhomme et al., 2016). Taken together, the IPTG-inducible CRISPRi system is highly specific.
We next used the CRISPRi system to construct an expression knockdown library of pneumococcal essential genes. An sgRNA to each of the 391 potentially essential genes was designed as described previously (Larson et al., 2013) (Dataset EV3). Based on the sgRNA-luc plasmid (Fig 2A), we tested two different cloning strategies to introduce the unique 20-nt base-pairing region for each gene: infusion cloning and inverse PCR (Ochman et al., 1988; Irwin et al., 2012; Larson et al., 2013) (Fig EV2A). For infusion cloning, we synthesized two complementary primers consisting of the 20-nt base-pairing region flanked by 15-nt overlap sequences. The two complementary primers were then annealed to form a duplex DNA fragment and cloned into the vector by the infusion reaction, followed by direct transformation into S. pneumoniae D39 strain DC123. With inverse PCR, we used a phosphorylated universal primer, together with a gene-specific primer to fuse the 20-nt base-pairing region into the vector by PCR, followed by blunt-end ligation and direct transformation into S. pneumoniae D39 strain DC123. We compared the efficiency of the two methods by creating sgRNA strains targeting the known essential gene folA (spd_1401). Depletion of folA causes a clear growth defect, which could thus be used to test the functionality of sgRNAfolA in transformants. We found that 79% of the transformants produced by infusion cloning had a growth defect upon dCas9 induction with IPTG (38 out of 48 colonies), whereas 26% of the transformants generated by inverse PCR..

**Construction and growth analysis of the CRISPRi library**

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**Figure 1. An IPTG-inducible CRISPRi system for tunable repression of gene expression in S. pneumoniae.**

A. dcas9 and sgRNA sequences were chromosomally integrated at two different loci, and expression was driven by an IPTG-inducible promoter (P_{lac}) and a constitutive promoter (P3), respectively. With addition of IPTG, dCas9 is expressed and guided to the target site by constitutively expressed sgRNA. Binding of dCas9 to the 5' end of the coding sequence of its target gene blocks transcription elongation. In the absence of IPTG, expression of dCas9 is tightly repressed, and transcription of the target gene can proceed smoothly.

B. Genetic map of CRISPRi luc reporter strain XL28. To allow IPTG-inducible expression, the lacI gene, driven by the constitutive PF6 promoter, was inserted at the non-essential prsA locus; luc, encoding firefly luciferase, driven by the constitutive P3 promoter was inserted into the intergenic sequence between gene loci spd_0422 and spd_0423; dcas9 driven by the IPTG-inducible P_{lac} promoter was inserted into the bgaA locus; sgRNA-luc driven by the constitutive P3 promoter was inserted into the CEP locus (between treR and amiF).

C. The CRISPRi system was tested in the luc reporter strain XL28. Expression of dcas9 was induced by addition of different concentrations of IPTG. Cell density (OD_{595}) and luciferase activity (shown as RLU/OD) of the bacterial cultures were measured every 10 min. The values represent averages of three replicates with SEM.

D. RNA-Seq confirms the specificity of the CRISPRi system in S. pneumoniae. RNA sequencing was performed on the luc reporter strain XL28 (panel B) with or without 1 mM IPTG. The dcas9 and luc genes are highlighted. Data were analyzed with T-REX and plotted as a volcano plot. P-value equals 0.05 is represented by the horizontal dotted line. Two vertical dotted lines mark the twofold changes.
showed a phenotype (12/46). Sequencing validated that transformants with a growth defect contained the correct sgRNA sequence. Considering the convenience and efficiency, we adopted the infusion cloning strategy for sgRNA cloning in this study. All sgRNA constructs were sequenced and we considered them genetically functional when the sgRNA did not contain more than 1 mismatch to the designed sgRNA and no mismatches in the first 14-nt prior to the PAM. Using this approach, after a single round of cloning and sequencing, we successfully constructed 348 unique sgRNA strains (see Materials and Methods). Note that we are still in the process of constructing the remaining 43 sgRNA strains, the failure of which is likely caused by technical reasons (e.g., incorrect oligonucleotides, poor oligo annealing, low transformation).

To examine the effects of CRISPRi-based gene silencing, growth was assayed both in the presence and absence of 1 mM IPTG for 18 h in real time by microtiter plate assays. Two types of growth phenotypes were defined and identified: a growth defect and increased lysis (Fig EV2B–E). As shown in Fig 2B, CRISPRi-based repression of transcription led to a growth defect in 230 genes, 48 genes showed increased lysis, including 24 that demonstrated both a growth defect and increased lysis, and 94 genes showed no defect (see Dataset EV1). In total, 254 out of 348 target genes (about 73%) repressed by CRISPRi showed growth phenotypes. Comparing the optical densities between the uninduced and induced cells at the time point at which uninduced cells reached an OD_{595} of ~0.1, 174 genes repressed by CRISPRi displayed a more than fourfold growth defect, and 254 genes showed a more than twofold growth defect (Fig 2C). To further validate the specificity of the CRISPRi system, CRISPRi strains targeting eight genes identified as essential and eight genes as dispensable by Tn-seq were included in the growth analysis. The selected dispensable genes are present as a monocistron or are in an operon with other...
non-essential genes. As shown in Fig EV3A, no apparent growth defects could be observed when these non-essential genes were targeted by CRISPRi, while repression of essential genes led to strong growth defects (Fig EV3B).

It should be noted that CRISPRi repression of dispensable genes that are cotranscribed with essential genes can lead to growth phenotypes (Appendix Fig S1), which is due to polar effect of CRISPRi system (Qi et al, 2013). Thus, some of the genes may be targeted multiple times in the CRISPRi library (in case of more than one essential gene within the operon). We also observed that after a lag phase, most CRISPRi knockdowns with growth phenotypes eventually grow out to the same final OD (Fig EV4A). Re-culturing these cells showed the absence of sensitivity to IPTG, indicative of the presence of suppressor mutations (Fig EV4A). Indeed, by sequencing the two key elements of the CRISPRi system, the sgRNA and dcas9, we found that most of the suppressor strains contain loss-of-function mutation in the dcas9 coding sequence (Fig EV4B). This is similar to observations made for the CRISPRi system in Bacillus subtilis (Zhao et al, 2016).

Phenotyping pneumococcal genes by combined CRISPRi and high-content microscopy

To test whether CRISPRi was able to place genes in a functional category and thereby allow us to identify previously uncharacterized genes with a function in cell envelope homeostasis, we first analyzed the effects of CRISPRi-based repression on cell morphology using 68 genes. These genes were selected as they represent different functional pathways and have been identified as essential or crucial for normal pneumococcal growth by Tn-seq studies (van Opijnen et al, 2009; van Opijnen & Camilli, 2012) and by displaying strong growth phenotypes in our CRISPRi assay (Fig 2B and C). The selected genes have been associated with capsule synthesis (three genes), transcription (four genes), cell division (six genes), translation (seven genes), teichoic acid biosynthesis (nine genes), cell membrane synthesis (11 genes), chromosome biology (14 genes), and peptidoglycan synthesis (14 genes) (Table 1). High-content microscopy of the CRISPRi knockdowns showed a good correlation between reported gene function and observed phenotype. The common features of the morphological changes caused by CRISPRi repression of genes belonging to the same functional categories are summarized in Table 1. Growth analysis and microscopy phenotyping of a representative gene of each pathway, CRISPRi repression of which showed typical morphological changes of its pathway, were included in Fig 3. Morphological changes of CRISPRi repression of the other genes of the pathways are shown in Appendix Figs S2–S9. For instance, compared with the control strain (Fig 3A, XL28), repression of transcription of genes involved in chromosome biology caused, as expected, appearance of anucleate cells or cells with aberrant chromosomes (Fig 3B, dnaA; Appendix Fig S2). Cells with repression of genes involved in transcription showed a significant growth defect, and no obvious morphological changes were observed (Fig 3C, rpoC; Appendix Fig S3). Repression of genes involved in translation showed heterogeneous cell shapes and condensed nucleoids (Fig 3D, infC; Appendix Fig S4), in line with our previous observations (Sorg & Veening, 2015) and observations made in Escherichia coli showing that inhibition of protein synthesis by antibiotics leads to nucleoid condensation (Morgan et al, 1967; Zusman et al, 1973; Riggiani & Goulain, 2015).

In S. pneumoniae, the fatty acid biosynthesis genes are all located in a single cluster (Lu & Rock, 2006) (Appendix Fig S5A), and two promoters in front of fabT and fabK are regulated by the transcriptional repressor FabT (Jerga & Rock, 2009). It was shown that fabT and fabH are cotranscribed (Lu & Rock, 2006), but the transcription pattern of the other genes is still unknown, which makes functional study of these genes with CRISPRi very difficult due to polar effects of the block of transcription elongation (Qi et al, 2013). Nevertheless, repression of transcription of genes involved in cell membrane synthesis caused diverse patterns of morphological changes: repression of fabH, acpP, fabK, fabD, and fabC led to a spotty Nile red pattern and irregular cell shapes including more pointy cells (Fig 3E, fabK; Appendix Fig S5B), as was shown previously (Kuiipers et al, 2016); repression of fabF, acxB, fabZ, and accD led to chaining of cells, heterogeneous cell sizes and irregular cell shapes; repression of acpS resulted in elongated and enlarged cells, whereas repression of cdsA caused cell rounding with heterogeneous cell sizes (Appendix Fig S5B).

When transcription of genes involved in capsule division was repressed, we observed cells with irregular shapes and heterogeneous sizes (Appendix Fig S6). Interestingly, repression of ftsZ and ftsL caused similar morphological changes (Fig 3F, ftsZ; Appendix Fig S6), consistent with the reported function of FtsZ on regulating FtsZ ring (Z-ring) dynamics in B. subtilis (Kawai & Ogasawara, 2006). Cells with repression of ezrA formed twisting chains and contained multiple septa, some of which formed at cell poles instead of midcell. Indeed, it was reported that B. subtilis EzrA can modulate the frequency and position of the Z-ring formation (Chung et al, 2004).

Repression of genes involved in capsule synthesis caused aggre- gation of cells (Appendix Fig S7), which may be due to the reduction in the negatively charged capsule that can provide a repelling electrostatic force preventing cell aggregation (Li et al, 2013).

Repression of transcription of genes involved in cell wall synthesis caused different phenotypes, depending on which step in peptidoglycan synthesis was interrupted. S. pneumoniae is oval-shaped, and it displays both septal and peripheral growth (Massidda et al, 2013; Pinho et al, 2013). Peptidoglycan synthesis of S. pneumoniae starts from formation of UDP-MurNAc-pentapeptides. Repression of expression of genes playing roles in these very first steps, including glmU, alr, ddl, murL, murC, murD, murE, and murF, will block both septal and peripheral peptidoglycan synthesis. Consistent with this prediction, we observed severe changes in cell shape and size, including heterogeneous cell sizes, exploding cells, defective septa, round cells, and cells demonstrating a coccus-to-rod transition (Appendix Fig S8). MraY and MurG play roles in formation of lipid II, and they are thus also involved in both peripheral and septal peptidoglycan synthesis. CRISPRi strains repressing mraY or murC led to a mix of elongated cells and short cells (Appendix Fig S8). FtsW and RodA are members of SEDS (shape, elongation, division, and sporulation) proteins (Meeske et al, 2016) and were first identified in E. coli (Ikeda et al, 1989). Inactivation of FtsW in E. coli blocks cell division without an effect on cell elongation (Khattar et al, 1994), and FtsW is suggested to act as a lipid II flippase (Mohammadi et al, 2011). FtsW of S. pneumoniae was believed to have a conserved function with E. coli (Maggi et al, 2008), is
co-localized with septal HMW (high molecular weight) PBPs (Morlot et al., 2004), and is thus predicted to be involved in septal peptidoglycan synthesis. By morphological analysis, we provided experimental evidence to support this prediction: FtsW and Pbp2X are responsible for septal peptidoglycan synthesis, and elongated cells and coccus-to-rod transition were observed with CRISPRi repression of ftsW or pbp2X (Fig 3G, pbp2X; Appendix Fig S8, ftsW). RodA of S. pneumoniae shows 26% identity with RodA of E. coli (Noirclerc-Savoye et al., 2003), which is required for cell elongation. Studies of RodA in B. subtilis also support its function on elongation of the lateral wall (Henriques et al., 1998; Meeske et al., 2016). RodA of S. pneumoniae was predicted to be a lipid II flippase responsible for peripheral peptidoglycan synthesis (Massidda et al., 2013). Streptococcus pneumoniae cells with repressed rodA expression by CRISPRi are consistently shorter (Appendix Fig S8), indicating a defect in cell elongation.

Table 1. Cellular pathways selected for CRISPRi phenotyping.

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<tr>
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<th>Phenotype</th>
<th>Gene*</th>
<th>Pathway</th>
<th>Phenotype</th>
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<tr>
<td>Cell membrane biosynthesis</td>
<td>Spotty membrane staining; irregular</td>
<td>cdaA (SPD_0244)</td>
<td>Teichoic acid biosynthesis</td>
<td>Longer chains; elongated cells;</td>
<td>spd_0099</td>
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<td></td>
<td>cell shape; heterogeneous cell size</td>
<td>fabH (SPD_0380)</td>
<td></td>
<td>enlarged cells; heterogeneous cell</td>
<td>licC (SPD_1123)</td>
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<tr>
<td></td>
<td></td>
<td>acpp (SPD_0381)</td>
<td></td>
<td>size; defective septa</td>
<td>licB (SPD_1124)</td>
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<tr>
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<td></td>
<td>fabK (SPD_0382)</td>
<td></td>
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<td>licA (SPD_1125)</td>
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<td>accP (SPD_1509)</td>
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*The genes highlighted in bold were included in Fig 3.
Figure 3.

Xue Liu et al  
CRISPRi phenotyping in *Streptococcus pneumoniae*  
Molecular Systems Biology 13:931 | 2017

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Repression of genes involved in teichoic acid (TA) biosynthesis led to morphological changes, including formation of longer chains and cells of heterogeneous sizes, mostly enlarged or elongated (Fig 3H, licD3, Appendix Fig S9). Growth of *S. pneumoniae* depends on exogenous choline, which is an essential molecule for the synthesis of pneumococcal TA, and the chaining phenotype caused by repression of genes involved in TA synthesis is in line with *S. pneumoniae* growing in medium without choline (Damjanovic et al., 2007).

In summary, by morphological analysis of CRISPRi strains for repression of transcription of genes with known function from different pathways, we established links between genotypes and phenotypes. Importantly, repression of transcription of genes known to be involved in cell envelope homeostasis, such as ftsZ, ftsL, ftsW, rodA, pbp2X, gilU, murC, murF, tarl, tar2, licA, licB, licC, and licD3, caused severe changes in cell shape and size, including heterogeneous cell size, ballooning cells, defective septa, short cells, round cells, in chains, and cells demonstrating a coccus-to-rod transition. These observations provide a useful platform for the functional identification of hypothetical genes, especially genes involved in cell envelope homeostasis.

**Functional verification and annotation of pscb (spd_2043), vicR (spd_1085), divIC (spd_0008), and rafX (spd_1672)**

We next analyzed 44 strains in the CRISPRi library that target genes that are annotated as hypothetical in the *S. pneumoniae* D39 genome in the NCBI database (CP000410.1, updated on 31-JAN-2015). From this approach, we were able to verify the function and annotate several genes, whose function had been studied in pneumococci before but have not been properly annotated in the D39 genome. For example, repression of genes (spd_0008, spd_1085, and spd_2043) led to significant growth defects and cell shape and cell size changes (Appendix Fig S11A and B). Knocking down *spd_2043* and *spd_1085* led to almost the same morphological changes, which included irregular cell shape, heterogeneous cell sizes, and appearance of ballooned cells, suggesting that these two genes might be functionally associated and play roles in peptidoglycan synthesis or cell division. By literature mining and BLAST searches, we recognized *spd_1085* as *vicR* and *spd_2043* as *pscb* (Ng et al., 2003). Consistent with the observed phenotypes in the CRISPRi strains, *pscb* was shown to be essential for cell wall separation and its expression relies on the response regulator encoded by *vicR* (Reinscheid et al., 2001; Ng et al., 2003; Sham et al., 2011; Bartual et al., 2014). Similarly, the morphological changes suggested a potential role of *SPD_0008* in cell wall synthesis or cell division. In line with this, *SPD_0008* was identified as *DivIC*, which was reported to form a trimeric complex with *DivIB* and *FtsL* and colocalized at division sites of *S. pneumoniae* strain R6 (Noircrlec-Savoye et al., 2005).

CRISPRi knockdown strain targeting *spd_1672* showed no significant growth defect at exponential phase, but cells lysed quicker in the stationary phase (Appendix Fig S11C). Microscopy showed that bacterial cells with CRISPRi-repressed *spd_1672* formed significantly longer chains (Appendix Fig S11D). Chained cells displayed irregular shapes and heterogeneous cell sizes. These phenotypes are very similar to the morphological changes caused by repression of genes involved in the biosynthesis of teichoic acid (Appendix Fig S9). Actually, *spd_1672* has been studied in *S. pneumoniae* R6 and was shown to contribute to the biosynthesis of wall teichoic acid and was named *rafX* (Wu et al., 2014). The reported *spd_1672* knockout strain of *S. pneumoniae* R6 also displayed a reduced stationary phase with similar cell shape and cell size defects. Inconsistent with our study, longer chains were not observed by TEM (transmission electron microscopy) imaging in the Wu et al study. To exclude the possible polar effect of CRISPRi repression, we made a *spd_1672* knockout in *S. pneumoniae* D39, and the *spd_1672* knockout strain also showed longer chains. Thus, the mismatch in phenotypes between the studies may be due to the different genetic background of *S. pneumoniae* D39 and R6, or may be caused by the process of sample preparation for TEM examination.

**Annotation and characterization of chromosome replication genes dnaB (spd_1522), dnaD (spd_1405), and yabA (spd_0827)**

High-content microscopy screening of the CRISPRi library showed that repression of *spd_1405*, *spd_1522*, and *spd_0827* led to significant growth defects and generation of anucleate cells (Appendix Fig S10). Appearance of anucleate cells is an important sign of a defect in chromosome biology, thus suggesting that these three genes are involved in chromosome replication or segregation. *SPD_0827* shows 33% identity with initiation control protein YabA of *Bacillus subtilis*, which interacts with DnaN and DnaA, and acts as a negative regulator of replication initiation (Noirnot-Gros et al., 2002; Goranov et al., 2009). We thus named *SPD_0827* to YabA. To test the function of *yabA* in *S. pneumoniae*, a deletion mutant was made by erythromycin marker replacement. The *yabA* deletion (*ΔyabA*) showed a significantly reduced growth rate compared to the wild type (Appendix Fig S12A) and displayed longer chains with frequent anucleate cells (Appendix Fig S12C). To test whether *S. pneumoniae* YabA is also a negative regulator of initiation of DNA replication, we determined the oriC-ter ratio using real-time quantitative PCR (qPCR). As shown in Appendix Fig S12D, the oriC-ter ratio was significantly higher in *ΔyabA* indicative of over-initiation, strongly suggesting a similar function as *B. subtilis* YabA.
When making a list of known genes involved in pneumococcal chromosome biology (Table 1), we noticed that dnaB and dnaD, two known bacterial DNA replication proteins (Smits et al., 2011; Briggs et al., 2012), are not annotated in S. pneumoniae D39. BlastP analyses showed that spd_1405 and spd_1522 might be coding for DnaD and DnaB, respectively. SPD_1405 showed 30% identity with DnaD of B. subtilis, and thus, we named spd_1405 to dnaD. SPD_1522 has 389 amino acids (aa), and the N-terminal 1–149 aa-long domain showed 19.8% identity with domain I of DnaB of B. subtilis, whereas aa 206–379 showed 45.7% identity with domain II. DnaB of B. subtilis (472 aa) is longer than SPD_1522 of S. pneumoniae D39 (389 aa), because the former contains a degenerated middle DDBH2 domain (Briggs et al., 2012). Additionally, the arrangement of the neighboring genes of S. pneumoniae dnaB (spd_1522), dnaA, and ndrR is the same in B. subtilis. Based on these observations, we named spd_1522 to dnaB.

It was reported that DnaD and DnaB are recruited to the chromosome by DnaA and play important roles in chromosome replication initiation in B. subtilis (Smits et al., 2011). To test the function of S. pneumoniae DnaD and DnaB, we constructed Zn2+-inducible depletion strains (PZn−dnaD; PZn−dnaB), because efforts to make deletion mutants failed. In the absence of 0.1 mM Zn2+, the depletion strains showed significant growth defects (Appendix Fig S12A), confirming their essentiality. If DnaB and DnaD indeed play a role in replication initiation, repression of them should lead to a decrease in the oriC-ter ratio. Indeed, the oriC-ter ratio of cells in absence of Zn2+ was significantly lower than in the presence of Zn2+ (Appendix Fig S12D). Together, we identified and annotated yabA, dnaD, and dnaB and confirmed their function in pneumococcal DNA replication.

SPD_1416 and SPD_1417 are involved in peptidoglycan precursor synthesis

We found that CRISPRi strains with sgRNA targeting hypothetical genes spd_1416 or spd_1417 showed significant growth retardation and morphological abnormality, such as heterogeneous cell size and elongated and enlarged cells with multiple incomplete septa (Appendix Fig S10). These manifestations mirrored what we observed upon inhibiting the expression of genes known to be involved in peptidoglycan (PG) synthesis (Appendix Fig S8). Consistent with the essentiality of these two genes as suggested by Tn-seq, we were unable to obtain deletion mutants of spd_1416 or spd_1417 after multiple attempts. To confirm that these genes are essential for pneumococcal growth, we constructed merodiploid strains of spd_1416 and spd_1417 by inserting a second copy of each gene fused to gfp (encoding a monomeric superfolder GFP) at their N-terminus (referred as gfp-spd_1416 or gfp-spd_1417) or C-terminus (referred as spd_1416-gfp or spd_1417-gfp). These gfp fusions were integrated at the ectopic bgaA locus under the control of the zinc-inducible promotor, Pz. In the presence of Zn2+, we could delete the native spd_1416 or spd_1417 gene by allelic replacement in the Pz-gfp-spd_1416 or Pz-gfp-spd_1417 genetic background. When transforming in the Pz-gfp-spd_1417-gfp genetic background, we did not obtain erythromycin resistant colonies, indicating that the C-terminal GFP fusion of SPD_1417 is not functional. Note that we could not replace spd_1416 or spd_1417 in the wild type in the presence of Zn2+. While both the spd_1416 and spd_1417 mutants behaved normally in the presence of Zn2+, severe growth retardation was observed in the absence of Zn2+ (Fig 4A). Together, these lines of evidence demonstrate that both spd_1416 and spd_1417 are essential genes.

Morphological analysis by light microscopy of bacterial cells upon depletion of gfp-spd_1416 or gfp-spd_1417 confirmed the morphological changes as observed in the CRISPRi knockdowns (Fig 4B). The gfp-spd_1416 or gfp-spd_1417 cells were further analyzed using freeze-substitution electron microscopy (Fig 4C). This showed the presence of elongated cells and the frequent formation of multiple septa per cell, in contrast to wild-type D39 cells which showed the typical diplococcal shape. Note that the mild sample preparation used in our freeze-substitution EM protocol also preserved the capsule, which can be readily lost during traditional EM sample preparation (Hammerschmidt et al., 2005). BlastP analysis shows that SPD_1416 contains a Mur-ligase domain with 36% sequence identity with MurT of Staphylococcus aureus, whereas SPD_1417 possesses a glutamine amidotransferase domain with 40% sequence identity with GatD of S. aureus. MurT and GatD, two proteins involved in staphylococcal cell wall synthesis (Figueiredo et al., 2012; Munch et al., 2012), form a complex to perform the amidation of the D-glutamic acid in the stem peptide of PG. It was previously reported that recombinant MurT/GatD of S. pneumoniae R6, purified from E. coli, indeed can amidate glutamate lipid II into iso-glutamine lipid II in vitro (Zapun et al., 2013). Therefore, we named spd_1416 to murT and spd_1417 to gatD. It is interesting to note that while MurT or GatD depletion strains in S. aureus showed reduced growth, cells exhibited normal cell morphologies (Figueiredo et al., 2012), in contrast to the strong morphological defects observed in S. pneumoniae D39.

MurT and GatD contain no membrane domain or signal peptide, and are thus predicted to be cytoplasmic proteins. However, fluorescence microscopy of the N-terminal GFP fused to MurT or GatD showed that they are partially membrane localized (Fig 4D). In-gel fluorescence imaging showed that GFP-MurT and GFP-GatD were correctly expressed without any detectable proteolytic cleavage (Appendix Fig S13). Since in vitro assays demonstrated that glutamate lipid II, which is anchored to the membrane by the bactoprenol hydrocarbon chain of lipid II, is a substrate of the MurT/GatD amidotransferase complex, it is reasonable to assume that membrane localization of MurT or GatD is caused by recruitment to the membrane-bound substrate. Indeed, amidation of the glutamic acid at position 2 of the peptide chain most likely occurs after formation of lipid-linked PG precursors (Rajagopal & Walker, 2016).

CRISPRi revealed novel pneumococcal genes involved in teichoic acid biosynthesis

CRISPRi-based repression of hypothetical essential genes spd_1197 and spd_1198 led to significant growth defects, and microscopy revealed chained cells with abnormal shape and size (Appendix Fig S10). Some of the cells were elongated and enlarged. These phenotypes are consistent with the typical morphological changes caused by repression of genes in teichoic acid (TA) biosynthesis (Appendix Fig S9). In accordance with this, analysis of the genetic context of spd_1197 and spd_1198 showed that they are in the lic3 region, which was predicted to be a pneumococcal TA gene cluster.
Similar to the approach described above, we generated Zn$^{2+}$-inducible C-terminal GFP fusions to SPD_1197 and SPD_1198, integrated these ectopically at the bgaA locus, and then deleted the native spd_1197 or spd_1198 genes in the presence of Zn$^{2+}$. Plate reader assays showed strong growth impairment in the absence of Zn$^{2+}$ (Fig 5A), suggesting their essentiality. In line with this, we were unable to replace these genes with an erythromycin resistance marker in the wild-type background in either the absence or presence of Zn$^{2+}$. Consistent with the phenotypes of the CRISPRi screen, the zinc-depletion strains showed similar morphological defects with cells in chains and elongated or enlarged cell shape and size (Fig 5B). EM analysis of depleted cells also revealed uneven distribution of multiple septa within a single cell, increased extracellular material and a rough cell surface (Fig 5C).

SPD_1198 contains 11 predicted transmembrane (TM) helices, while SPD_1197 has 2 predicted TM segments with a C-terminal extracytoplasmic tail. In-gel fluorescence showed that SPD_1197-GFP was mainly produced as a full-length product. The SPD_1198-GFP fusion, however, showed clear signs of protein degradation (Appendix Fig S13). Nevertheless, we performed fluorescence microscopy to determine their localizations. In agreement with the prediction, SPD_1197-GFP and SPD_1198-GFP are clearly localized to the membrane (Fig 5D).

Phosphorylcholine is an essential component of pneumococcal TA, and for this reason, a phosphorylcholine antibody is frequently used to detect S. pneumoniae TA (Vollmer & Tomasz, 2001; Wu et al, 2014). To explore whether SPD_1197 and SPD_1198 indeed play a role in TA synthesis, we performed Western blotting to detect phosphorylcholine-decorated TA using whole-cell lysates (Fig 5E). Cells of strains P_Zn^-spd_1197-gfp and P_Zn^-spd_1198-gfp were grown in the presence or absence of 0.1 mM Zn$^{2+}$. As controls, we depleted expression of three genes involved in PG synthesis (murT, gatD, and pbp2x). As shown in Fig 5E, Zn$^{2+}$ did...
not influence TA synthesis of the *S. pneumoniae* D39 wild-type (WT) strain, and the four main TA bands are clearly visible, migrating in the range between 15 and 25 kDa consistent with previous reports (Wu et al., 2014). In contrast, cells depleted for SPD_1197 or SPD_1198 displayed a different pattern and the 4 main bands around 15 and 25 kDa were missing or much weaker,
while multiple bands with a size smaller than 15 kDa appeared. TA of *S. pneumoniae*, including wall teichoic acid (WTA) and membrane-anchored lipoteichoic acid (LTA), are polymers with identical repeating units (RU) (Fischer et al., 1993). Addition of one RU can lead to about a 1.3 kDa increase in molecular weight (Gisch et al., 2013). Interestingly, the weight interval between the extra smaller bands from bacterial cells with depleted SPD_1197 or SPD_1198 seemed to match the molecular weight of the RU, suggesting that SPD_1197 and SPD_1198 play a role in TA precursor polymerization. Although repression of the genes associated with peptidoglycan synthesis (*murT*, *gatD*, and *pbp2x*) made the 4 main TA bands weaker, the pattern of the TA bands was not changed. Likely, the reduction in the TA of these three strains is due to the reduction in peptidoglycan, which constitutes the anchor for wall TA. Additionally, a CRISPRi strain targeting *tarI* of the *lic1* locus, which is involved in an early step of TA precursor synthesis, was included as a control. Note that *tarI* is cotranscribed with the other four genes of the *lic1* locus, including *tarJ*, *licA*, *licB*, and *licC*. Likely, CRISPRi knockdown of *tarI* will repress transcription of the entire *lic1* locus and thus block the synthesis of TA precursors. In line with this, we observed a reduction in the total amount of teichoic acid chains when *tarI* was repressed by CRISPRi (Appendix Fig S14).

The TA chains of *S. pneumoniae* are thought to be polymerized before they are transported to the outside of the membrane by the flippase TacF (Damjanovic et al., 2007), and so far it is not known...
which protein(s) function(s) as TA polymerase (Denapaite et al., 2012). In line with SPD_1198 being the TA polymerase, homology analysis shows that it contains a predicted polymerase domain. The large cytoplasmic part of SPD_1197 may aid in the assembly of the TA biosynthetic machinery by protein–protein interactions (Denapaite et al., 2012). Together, we here show that SPD_1197 and SPD_1198 are essential for growth and we suggest that they are responsible for polymerization of TA chains (Fig 5F). Consistent with the nomenclature used for genes involved in TA biosynthesis, we named spd_1198 tarP (for teichoic acid ribitol polymerase) and spd_1197 tarQ (in operon with tarP, sequential alphabetical order). Whether TarP and TarQ interact and function as a complex remains to be determined.

The essential ATPase ClpX and the protease ClpP repress competence development

We wondered whether we could also employ CRISPRi to probe gene regulatory networks in which essential genes play a role. An important pathway in S. pneumoniae is development of competence for genetic transformation, which is under the control of a well-studied two-component quorum sensing signaling network (Claverys et al., 2009). Several lines of evidence have shown that the highly conserved ATP-dependent Clp protease, ClpP, in association with an ATPase subunit (either ClpC, ClpE, ClpL, or ClpX), is involved in regulation of pneumococcal competence (Charpentier et al., 2000; Chastanet et al., 2001) (Fig 6A). Identification of the ATPase subunit responsible for ClpP-dependent repression of competence was hampered because of the essentiality, depending on the growth medium and laboratory conditions, of several clp mutants including clpP and clpX (Chastanet et al., 2001). To address this issue, we employed CRISPRi and constructed sgRNAs targeting clpP, clpC, clpE, clpL, and clpX. Competence development was quantified using a luc construct, driven by a competence-specific promoter (Slager et al., 2014). As shown in Fig 6B, when expression of ClpP or ClpX was repressed by addition of IPTG, competence development was enhanced, while depleting any of the other ATPase subunits (ClpC, ClpE, and ClpL) had no effect on competence (Figs 6C and EV5). This shows that ClpX is the main ATPase subunit responsible for ClpP-dependent repression of competence.

Discussion

Here, we developed an IPTG-inducible CRISPRi system to study essential genes in S. pneumoniae (Fig 1). In addition, we adopted a simple and efficient one-step sgRNA engineering strategy using infusion cloning. This approach resulted in ~89% positive sgRNA clones after a single round of transformation, thus enabling high-throughput cloning of sgRNAs.

Growth analysis of the CRISPRi strains targeting the 348 potentially essential genes showed that individual repression of 73% of the targeted genes led to growth phenotypes, using a stringent cutoff for phenotype detection (Figs 2B and C, and EV2). There could be several reasons why CRISPRi knockdown of the remaining 94 genes did not cause a detectable growth phenotype. Tn-seq sometimes incorrectly assigns an essential function to non-essential genes (van Opijnen et al., 2009; van Opijnen & Camilli, 2013). Also, Tn-seq relies on a round of growth on blood agar plates, while our CRISPRi phenotypes were only assayed in liquid C–Y medium. Additionally, we used stringent cutoffs for phenotype definition, which will miss genes with mild growth or lysis phenotypes. Certain genes might also not be repressed well enough by CRISPRi to show a phenotype (in case of stable proteins that only require a few molecules for growth). This can be for instance caused when the sgRNA targets a PAM site far away from the transcription start site, when there is poor access of the sgRNA-dCas9 complex to the target DNA or when there are polar effects within the operon alleviating the essentiality. We can also not exclude a suppressor mutation arising in some of the “No phenotype” CRISPRi strains, as most CRISPRi knockdowns with growth phenotypes eventually grew out to the same final OD and contain a loss-of-function mutation in the coding sequence of dcas9 (Fig EV4).

Based on analysis of the CRISPRi knockdowns, several previously “hypothetical” genes could be functionally characterized and annotated. For instance, combined with BlastP analysis and determination of oric-ter ratios, we could annotate the pneumococcal primosomal machinery, including DnaA, DnaB, DnaC, DnaD, DnaG, and Dnal (Table 1, Appendix Figs S2 and S12). Note that spd_2030 (dnaC) was mis-annotated as dnaB in several databases, such as in NCBI (ProteinID: ABJ54728), KEGG (Entry: SPD_2030), Uniprot (Entry: A0A0H2ZNF7), which may be due to the different naming of primosomal proteins in E. coli and Bacillus subtilis (Smits et al., 2011; Briggs et al., 2012). By characterizing CRISPRi-based knockdowns with cell morphology defects, we identified four essential cell wall biosynthesis genes (murt, gatD, tarP, and tarQ), which are promising candidates for future development of novel antimicrobials.

This work and other studies highlight that high-throughput phenotyping by CRISPRi is a powerful approach for hypothesis-forming and functional characterization of essential genes (Peters et al., 2016). We also show that CRISPRi can be used to unravel gene regulatory networks in which essential genes play a part (Fig 6). While we shed light on the function of just several previously uncharacterized essential genes, the here-described library contains richer information that needs to be further explored. In addition, CRISPRi screens can be used for mechanism of action (MOA) studies with new bioactive compounds. Indeed, CRISPRi was recently successfully employed to show that B. subtilis UppS is the molecular target of compound MAC-0170636 (Peters et al., 2016). We anticipate that the here-described pneumococcal CRISPRi library can function as a novel drug target discovery platform, can be applied to explore host–microbe interactions, and will provide a useful tool to increase our knowledge concerning pneumococcal cell biology.

Materials and Methods

Strains, growth conditions, and transformation

Oligonucleotides are shown in Dataset EV4 and strains in Appendix Table S1. Streptococcus pneumoniae D39 and its derivatives were cultivated in C–Y medium, pH = 6.8 (Slager et al., 2014) or Columbia agar with 2.5 % sheep blood at 37°C. Transformation of S. pneumoniae was performed as previously described (Martin et al., 2000), and CSP-1 was used to induce competence.
Transformants were selected on Columbia agar supplemented with 2.5% sheep blood at appropriate concentrations of antibiotics (100 µg/ml spectinomycin, 250 µg/ml kanamycin, 1 µg/ml tetracycline, 40 µg/ml gentamycin, 0.05 µg/ml erythromycin). For construction of deletion strains with the Zn2+-inducible promoter, 0.1 mM ZnCl2/0.01 mM MnCl2 was added to induce the ectopic copy of the target gene (mentioned as 0.1 mM Zn2+ for convenience). Working stock of the cells, called “T2 cells”, were prepared by growing the cells in C+Y medium to OD600 0.4, and then resuspending the cells with equal volume of fresh medium with 17% glycerol.

*Escherichia coli* MC1061 was used for subcloning of plasmids, and competent cells were prepared by CaCl2 treatments. The *E. coli* transformants were selected on LB agar with appropriate concentrations of antibiotics (100 µg/ml spectinomycin, 100 µg/ml ampicillin, 50 µg/ml kanamycin).

**Construction of an IPTG-inducible CRISPRi system in *S. pneumoniae***

*Streptococcus pyogenes* dcas9 (*dcas9sp*) was obtained from Addgene (Addgene #44249, Qi et al, 2013) and subcloned into plasmid pJWV102 (Veening laboratory collection) with the IPTG-inducible promoter P_lac (Sorg, 2016) replacing P têm, resulting in plasmid pJWV102-P_lac-dcas9sp. pJWV102-P_lac-dcas9sp was integrated into the bgaA locus in *S. pneumoniae* D39 by transformation. To control P_lac expression, a codon-optimized *E. coli lacI* gene driven by the constitutive promoter PF6 was inserted at the *prsA* locus in *S. pneumoniae* D39 (Sorg, 2016), leading to the construction of strain DC123. DCI23 was used as the host strain for the insertion of genespecific sgRNAs and enables the CRISPRi system. The DNA fragment encoding the single-guide RNA targeting luciferase (sgRNA_luc) was ordered as a synthetic DNA gBlock (Integrated DNA Technologies) containing the constitutive P3 promoter (Sorg et al, 2015). The sgRNA_luc sequence is transcribed directly after the +1 of the promoter and contains 19 nucleotides in the base-pairing region, which binds to the non-template (NT) strand of the coding sequence of luciferase, followed by an optimized single-guide RNA (Chen et al, 2013) (Fig EV1A). Then, the sgRNA_luc with P3 promoter was cloned into pPEP1 (Sorg et al, 2015) with removing the chloramphenicol resistance marker (*pPEP1*) leading to the production of plasmid pPEPX-P3-sgRNA_luc, which integrates into the region between *antiF* and *treR* of *S. pneumoniae* D39. The pPEPX-P3-sgRNA_luc is used as the template for generation of other sgRNAs by infusion cloning or by the inverse PCR method. The *lacI* gene with gentamycin resistance marker and flanked *prsA* regions was subcloned into pPEPY (Veening laboratory collection), resulting in plasmid pPEPY-PF6-lacI. This plasmid can be used to amplify *lacI* and integrate it at the *prsA* locus while selecting for gentamycin resistance. The entire pneumococcal CRISPRi system, consisting of plasmids pJWV102-P_lac-dcas9sp, pPEPY-PF6-lacI, and pPEPX-P3-sgRNA_luc, is available from Addgene (ID 85588, 85589, and 85590, respectively).

**Selection of essential genes**

To identify each gene’s contribution to fitness for basal level growth, we performed Tn-seq in *S. pneumoniae* D39 essentially as described before (Zomer et al, 2012; Burghout et al, 2013), but with growing cells in C+Y medium at 37°C. Possibly essential genes were identified using ESSENTIALS (Zomer et al, 2012). Based on that, we included all the identified essential genes and added extra essential genes identified in serotype 4 strain TIGR4 (van Opipjen et al, 2009; van Opipjen & Camilli, 2012). Note that in the Tn-seq study of 2012, fitness of each gene under 17 in vitro and 2 in vivo conditions was determined and genes were grouped into different classes (van Opipjen & Camilli, 2012). Finally, 391 genes were selected (Dataset EV1).

**Oligonucleotides for the CRISPRi library**

The 20-nt guide sequences of the sgRNAs targeting different genes were selected with CRISPR Primer Designer (Yan et al, 2015). Briefly, we searched within the coding sequence of each essential gene for a 14-nt specificity region consisting of the 12-nt “seed” region of the sgRNA and GG of the 3-nt PAM (GGN). sgRNAs with more than one binding site within the pneumococcal genome, as determined by a BLAST search, were discarded. Next, we took a total length of 21 nt (including the +1 of the P3 promoter and 20 nt of perfect match to the target) and the full-length sgRNA’s secondary structure was predicted using ViennaRNA (Lorenz et al, 2011), and the sgRNA sequence was accepted if the dCas9 handle structure was folded correctly (Larson et al, 2013). We chose the guide sequences as close as possible to the 5’ end of the coding sequence of the targeted gene (Qi et al, 2013). The sequences of the sgRNAs (20 nt) are listed in Dataset EV3.

**Cloning of sgRNA**

We used infusion cloning instead of inverse PCR recommended by Larson et al (2013) because significantly higher cloning efficiencies were obtained with infusion cloning. Two primers, sgRNA_inF_plasmid_linearize_R and sgRNA_inF_plasmid_linearize_F, were designed for linearization of plasmid pPEPX-P3-sgRNA_luc. These two primers bind directly upstream and downstream of the 19-bp guide sequence for *lac*. To fuse the 20-nt new guide sequence into the linearized vector, two 50-nt complementary primers were designed for each target gene. Each primer contains 15 nt at one end, overlapping with the sequence on the 5’ end of the linearized vector, followed by the 20-nt specific guide sequence for each target gene; and 15 nt overlapping with the sequence on the 3’ end of the linearized vector (Fig EV2A). The two 50-nt complementary primers were annealed in TEN buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8) by heating at 95°C for 5 min and cooling down to room temperature. The annealed product was fused with the linearized vector using the Quick-Fusion Cloning kit (BiMake, Cat. B22612) according to the manufacturer with the exception of using only one half of the recommended volume per reaction. Each reaction was directly used to transform competent *S. pneumoniae* D39 strain DCI23.

**Luciferase assay**

*Streptococcus pneumoniae* strains XL28 and XL29 were grown to OD600 = 0.4 in 5-ml tubes at 37°C and then diluted 1:100 in fresh C+Y medium with or without 1 mM IPTG. Then, in triplicates, 250-µl diluted bacterial culture was mixed with 50 µl of 6× luciferin solution in C+Y medium (2.7 mg/ml, D-Luciferin sodium salt, 0.4 in 5-ml tubes at 37°C and then diluted 1:100 in fresh C+Y medium with or without 1 mM IPTG. Then, in triplicates, 250-µl diluted bacterial culture was mixed with 50 µl of 6× luciferin solution in C+Y medium (2.7 mg/ml, D-Luciferin sodium salt,
SYNChem OHG) in 96-well plates (Polystyrol, white, flat, and clear bottom; Corning). Optical density at 395 nm (OD595) and luminescence signal were measured every 10 min for 10 h using a Tecan Infinite F200 Pro microtiter plate reader.

Growth assays

For growth curves of strains of the CRISPRi library, T2 cells were thawed and diluted 1:1,000 into fresh C+Y medium with or without 1 mM IPTG. Then, 300 μl of bacterial culture was added into each well of 96-well plates. OD595 was measured every 10 min for 18 h with a Tecan Infinite F200 Pro microtiter plate reader. Specially, for the data shown in Fig 3, Appendix Figs S10 and S11, T2 cells were diluted 1:100 in C+Y medium. For growth assays of the depletion strains with the Zn2+-inducible promoter, T2 cells were thawed and diluted 1:100 into fresh C+Y medium with or without 0.1 mM Zn2+.

Detection of teichoic acids

Sample preparation

T2 cells of S. pneumoniae strains were inoculated into fresh C+Y medium with 0.1 mM Zn2+ by 1:50 dilution, and then grown to OD600 0.15 at 37°C. Cells were collected at 8,000 g for 3 min and resuspended with an equal volume of fresh C+Y medium without Zn2+. Bacterial cultures were diluted 1:10 into C+Y with or without 0.1 mM Zn2+ or 1 mM IPTG (for CRISPRi strains) and then incubated at 37°C. When OD600 reached 0.3, cells were centrifuged at 8,000 g for 3 min. The pellets were washed once with cold TE buffer (10 mM Tris–Cl, pH 7.5; 1 mM EDTA, pH 8.0), and resuspended with 150 μl of TE buffer. Cells were lysed by sonication.

Detection of teichoic acid with phosphoryl choline antibody

Mab TEPC-15

Protein concentration of the whole-cell lysate was determined with the DC protein assay kit (Bio-Rad Cat. 500-0111). Whole-cell lysates were mixed with equal volumes of 2× SDS protein loading buffer (100 mM Tris–HCl, pH 6.8; 4% SDS; 0.2% bromophenol blue; 20% glycerol; 10 mM DTT) and boiled at 95°C for 5 min. 2 μg of protein was loaded, followed by SDS–PAGE on a 12% polyacrylamide gel with cathode buffer (0.1 M Tris, 0.1 M tricine, 0.1% SDS) on top of the wells and anode buffer (0.2 M Tris/Cl, pH 9.9) in the bottom. After electrophoresis, samples in the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane as described (Minnen et al., 2011). Teichoic acid was detected with anti-PC specific monoclonal antibody TEPC-15 (M1421, Sigma) by 1:1,000 dilution as first antibody, and then with anti-mouse IgG HRP antibody (GE Healthcare UK Limited) with 1:5,000 dilution as second antibody. The blots were developed with ECL prime Western blotting detection reagent (GE Healthcare UK Limited), and the images were obtained with a Bio-Rad imaging system.

Microscopy

To detect the morphological changes after knockdown of the target genes, strains in the CRISPRi library were induced with IPTG and depletion strains were incubated in C+Y medium without Zn2+, stained with DAPI (DNA dye) and Nile red (membrane dye), and then studied by fluorescence microscopy. Specifically, 10 μl of thawed T2 cells was added into 1 ml of fresh C+Y medium, with or without 1 mM IPTG, in a 1.5-ml Eppendorf tube, followed by 2.5 h of incubation at 37°C. After that, 1 μl of 1 mg/ml Nile red was added into the tube and cells were stained for 4 min at room temperature. Then, 1 μl of 1 mg/ml DAPI was added and the mix was incubated for one more minute. Cells were spun down at 8,000 g for 2 min, and then, the pellets were suspended with 30 μl of fresh C+Y medium. 0.5 μl of cell suspension was spotted onto a PBS agarose pad on microscope slides. DAPI, Nile red, and phase contrast images were acquired with a Deltavision Elite (GE Healthcare, USA). Microscopy images were analyzed with ImageJ.

For fluorescence microscopy of strains containing zinc-inducible GFP fusions, strains were grown in C+Y medium to OD600 0.1, followed by 10 times dilution in fresh C+Y medium with 0.1 mM Zn2+. After 1 h of incubation, cells were spun down, washed with PBS, and resuspended in 50 μl PBS. 0.5 μl of cell suspension was spotted onto a PBS agarose pad on microscope slides. Visualization of GFP was performed as described previously (Kjos et al., 2015).

For electron microscopy, T2 cells of S. pneumoniae strains were inoculated into C+Y medium with 0.1 mM Zn2+ and incubated at 37°C. When OD600 reached 0.15, the bacterial culture was centrifuged at 8,000 g for 2 min. The pellets were resuspended into C+Y without Zn2+ such that OD600 was 0.015, and then, cells were incubated again at 37°C. Bacterial cultures were put on ice to stop growth when OD600 reached 0.35. Cells were collected by centrifugation and washed once with distilled water. A small pellet of cells was cryo-fixed in liquid ethane using the sandwich plunge freezing method (Baba, 2008) and freeze-substituted in 1% osmium tetroxide, 0.5% uranyl acetate, and 5% distilled water in acetone using the fast low-temperature dehydration and fixation method (McDonald & Webb, 2011). Cells were infiltrated overnight with Epon 812 (Serva, 21045) and polymerized at 60°C for 48 h. 90-nm-thick sections were cut with a Reichert ultramicrotome and imaged with a Philips CM12 transmission electron microscope running at 90 kV.

Competence assays

The previously described ssbB_luc competence reporter system, amplified from strain MK134 (Slager et al., 2014), was transformed into the CRISPRi strains (sgRNAclpP, sgRNAclpX, sgRNAclpL, sgRNAclpE, sgRNAclpC). Luminescence assays for detection of activation of competence system were performed as previously described (Slager et al., 2014). IPTG was added into C+Y medium (at a non-permissive pH for competence development) at the beginning of cultivation to different final concentrations.

Data availability

Raw Tn-seq data are available at the SRA with accession number SRR5298192; the RNA-Seq data are available at the GEO database with accession number GSE89763.

Expanded View for this article is available online.

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Author contributions

XL and J-WV designed the study. XL, MK, AD, CG, SPK, JS, and JWV performed experiments and analyzed the data. KK performed electron microscopy. RAS developed the IPTG-inducible system. JS analyzed the RNA-Seq data and XL and J-WV designed the study. XL, MK, AD, CG, SPK, JS, and JWV performed the CRISPRi growth analysis. XL, JS, MK, AD, CG, J-RZ, and J-WV wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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