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RESEARCH ARTICLE

CRISPR Interference Screens Identify Novel Regulators of Bacterial Biofilm Formation

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ABSTRACT

Bacterial biofilms underlie a large share of chronic and device-associated infections and are intrinsically tolerant of antibiotics that readily kill the same organism growing planktonically, yet the regulatory network governing the transition from free-living to surface-attached, matrix-encased growth remains incompletely mapped even in well-studied species. We used a pooled, genome-wide CRISPR interference (CRISPRi) library targeting 5,570 annotated genes in *Pseudomonas aeruginosa* PAO1 to screen for regulators of biofilm formation, selecting for sgRNA abundance changes between the planktonic and biofilm-associated bacterial fractions of a static microtiter biofilm assay after 24 hours. The screen identified 143 genes whose knockdown significantly altered biofilm formation, of which 41 were annotated with no prior functional link to biofilm or surface-sensing biology. Individual knockdown validation of the twelve highest-confidence novel hits confirmed a biofilm phenotype for nine, including PA2782, a previously uncharacterised two-component sensor histidine kinase whose knockdown reduced biofilm biomass by 68% relative to a non-targeting control without affecting planktonic growth rate. RNA sequencing of the PA2782 knockdown strain showed downregulation of the *pel* and *psl* exopolysaccharide biosynthesis operons and of the c-di-GMP-synthesising diguanylate cyclase gene PA2870, placing PA2782 upstream of the canonical c-di-GMP-dependent biofilm matrix pathway. The PA2782 knockdown strain also showed a 5.4-fold increase in tobramycin susceptibility within an established biofilm, consistent with reduced matrix production restoring antibiotic penetration. These results expand the known regulatory network controlling *P. aeruginosa* biofilm formation and identify PA2782 as a candidate target for biofilm-disruptive adjunct therapy.

Keywords: CRISPR interference; *Pseudomonas aeruginosa*; biofilm formation; genetic screen; quorum sensing; antibiotic tolerance

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1. INTRODUCTION

Pseudomonas aeruginosa is a leading cause of chronic lung infection in people with cystic fibrosis, of ventilator-associated pneumonia, and of infections associated with indwelling medical devices, and in each

of these settings the organism persists predominantly as a surface-attached biofilm rather than as free-swimming planktonic cells [1,2]. Biofilm growth confers tolerance to antibiotic concentrations that are readily bactericidal against the same strain grown planktonically, through a combination of restricted drug penetration through the

exopolysaccharide matrix, metabolically dormant subpopulations, and biofilm-specific stress responses, and this tolerance, rather than classical resistance mutation, is thought to explain much of the difficulty in clearing chronic *P. aeruginosa* infection with conventional antibiotic therapy alone [3,14]. Understanding the regulatory circuitry that commits a cell to biofilm growth, and identifying targets that could be used to disrupt or prevent that commitment, has therefore attracted sustained interest as a complementary strategy to antibiotic therapy itself.

The core biochemical logic of biofilm matrix production in *P. aeruginosa* is well established: the second messenger bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) accumulates in response to surface contact and other cues, activating transcription and post-translational production of the Pel and Psl exopolysaccharides and of extracellular DNA and CdrA adhesin that together form the biofilm matrix [4,15]. What remains considerably less complete is the upstream sensory and regulatory network that determines when and how strongly c-di-GMP synthesis is activated: the *P. aeruginosa* genome encodes more than 40 proteins with GGDEF or EAL diguanylate cyclase or phosphodiesterase domains, the enzymes that synthesise and degrade c-di-GMP respectively, and a comparable number of two-component sensor histidine kinases and response regulators of uncertain or only partially characterised function, leaving considerable scope for regulators that have simply not yet been systematically tested [5,16].

Forward genetic screens using transposon mutagenesis identified many of the biofilm regulators known today, but transposon approaches are poorly suited to essential or fitness-relevant genes, whose disruption is lethal or strongly growth-impairing regardless of any separate role in biofilm regulation, and to genes with partially redundant paralogues, where a single transposon insertion may produce little phenotype [6,17]. CRISPR interference, using a catalytically dead Cas9 (dCas9) targeted by a single guide RNA to block transcription without introducing a double-strand break or gene deletion, offers two practical advantages over transposon mutagenesis for this purpose: knockdown strength can be tuned by guide position and induction level rather than being all-or-nothing, and pooled CRISPRi libraries can be constructed with multiple independent guides per gene at a scale and uniformity that is difficult to achieve with transposon mutagenesis, improving statistical power to detect genuine hits against screen noise [7,8,18]. A further practical advantage, less often emphasised, is that a pooled CRISPRi library is comparatively straightforward to reuse across multiple selection conditions once constructed, since the same plasmid pool can be re-transformed or re-conjugated into a fresh recipient background and subjected to a different selective regime, whereas a transposon mutant library typically requires either an entirely new mutagenesis

campaign or careful archival and re-arraying of an existing ordered collection to achieve the same flexibility. This matters for biofilm biology specifically, where the field has increasingly recognised that regulatory circuitry identified under one assay format, such as a static microtiter plate, does not always transfer cleanly to a flow-cell or in-vivo infection model, and a reusable library lowers the practical barrier to testing whether a given hit's relevance is assay-specific or more generally robust across cultivation formats [36].

We used a pooled, genome-wide CRISPRi library targeting essentially all annotated *P. aeruginosa* PAO1 genes, under partial, sub-lethal induction to retain viability even for growth-relevant targets, to screen systematically for genes whose knockdown alters biofilm formation in a standard static microtiter assay. Our aim was not simply to recover already-known matrix and c-di-GMP pathway genes, which would mainly serve as an internal positive control, but specifically to identify genes with no prior annotated link to biofilm biology, and to characterise at least one such novel regulator mechanistically and functionally in enough depth to judge whether it represents a genuine addition to the biofilm regulatory network rather than a screen artefact or an indirect growth-rate effect.

2. MATERIALS AND METHODS

All experiments used *Pseudomonas aeruginosa* strain PAO1 and a genome-scale CRISPRi library constructed in this background using a chromosomally integrated, anhydrotetracycline-inducible dCas9 cassette and a pooled sgRNA plasmid library targeting the non-template strand of 5,570 annotated open reading frames with a mean of four independent guides per gene.

2.1 CRISPRi library construction and screening

The sgRNA library was synthesised as an oligonucleotide pool, cloned into a mobilisable CRISPRi plasmid backbone, and introduced into the dCas9-expressing PAO1 background by conjugation at a multiplicity calculated to yield fewer than one plasmid per recipient cell, preserving a clonal genotype-to-phenotype relationship. The pooled library, comprising approximately 22,000 unique sgRNA constructs, was grown under selection to an representation of at least 200-fold coverage per guide before splitting into screen replicates. dCas9 expression was induced with a sub-lethal concentration of anhydrotetracycline (10 ng/mL) chosen in pilot experiments to produce partial knockdown with minimal effect on planktonic growth rate across a panel of essential-gene-targeting control guides.

2.2 Static biofilm selection assay

Induced library cultures were seeded into 96-well polystyrene microtiter plates and incubated statically for 24 hours at 37 degrees C. Planktonic (unattached) bacteria were removed and retained as the planktonic fraction; adherent biofilm bacteria were recovered by

vigorous resuspension in phosphate-buffered saline following removal of the planktonic fraction and a single gentle wash step. Genomic DNA was extracted from both fractions across three independent screen replicates, and the integrated sgRNA cassette was amplified by PCR and sequenced on an Illumina platform to quantify sgRNA abundance in each fraction.

2.3 Screen analysis and hit calling

Guide-level counts were normalised to total reads per sample, and a gene-level enrichment or depletion score was calculated as the log₂ fold-change in mean guide abundance between the biofilm and planktonic fractions, combined across the four guides per gene using a rank-based aggregation statistic with an associated false discovery rate estimated by permutation. Genes with FDR<0.05 and an absolute log₂ fold-change greater than 1.0 in at least two of three screen replicates were called as significant hits. Hits were cross-referenced against the *Pseudomonas* Genome Database functional annotation to classify each as previously linked, or not previously linked, to biofilm, surface-sensing, or c-di-GMP signalling biology.

2.4 Individual validation and mechanistic follow-up

The twelve highest-confidence novel hits were reconstructed as individual CRISPRi knockdown strains, each carrying a single validated sgRNA against the target gene, and assayed for biofilm biomass by crystal violet staining and for planktonic growth rate by optical density time course, alongside a non-targeting sgRNA control. For the strongest validated hit, PA2782, RNA sequencing was performed on knockdown and control strains grown under biofilm-inducing conditions, and biofilm antibiotic susceptibility was assessed by measuring the minimum biofilm eradication concentration of tobramycin against 24-hour static biofilms of each strain.

2.5 Statistical analysis

Crystal violet biofilm biomass and growth rate comparisons between individual knockdown strains and the non-targeting control were analysed by one-way ANOVA with Dunnett's test. RNA sequencing differential expression was analysed using a standard negative-binomial generalized linear model with Benjamini-Hochberg correction for multiple testing. All analyses were performed in R version 4.3, with P<0.05, or FDR<0.05 for genome-wide comparisons, taken as the significance threshold throughout.

3. RESULTS

Across three independent screen replicates, sgRNA representation remained above the pre-specified 200-fold coverage threshold throughout the selection, and replicate biofilm-versus-planktonic fold-change estimates were well correlated (pairwise Pearson *r* between 0.71 and 0.79), indicating reasonable screen reproducibility. 143 genes met the significance threshold

for altering biofilm formation upon knockdown, comprising 97 whose knockdown reduced biofilm formation (candidate positive regulators) and 46 whose knockdown increased it (candidate negative regulators). As an internal validation of screen performance, the hit list recovered the *pel* and *psl* operons, the master regulator gene *retS*, and thirteen of the previously characterised GGDEF-domain diguanylate cyclase genes with an established positive role in biofilm formation, together accounting for 34 of the 143 hits and confirming that the screen was capturing genuine biofilm-regulatory biology rather than noise. Table 1 summarises the screen's hit-calling statistics and functional annotation breakdown.

Table 1. CRISPRi screen hit-calling summary and functional annotation breakdown.

Category	Number of genes	Example genes	FDR threshold
Positive regulators (KD reduces biofilm)	97	<i>pel</i> operon, <i>psl</i> operon, <i>retS</i>	<0.05
Negative regulators (KD increases biofilm)	46	<i>pilA</i> , <i>bdIA</i> , <i>rpoS</i>	<0.05
Previously known biofilm-linked genes	102	<i>pelA</i> , <i>pslA</i> , <i>retS</i> , <i>wspR</i>	--
Novel candidate regulators	41	PA2782, PA2783, PA0575	<0.05

Screen conducted across three independent replicates; hits required FDR<0.05 and |log₂FC|>1.0 in ≥2 of 3 replicates.

Of the 143 significant hits, 41 had no prior annotated functional link to biofilm formation, surface sensing, or c-di-GMP signalling in the *Pseudomonas* Genome Database, and were classified as candidate novel regulators. Individual reconstruction and validation of the twelve highest-confidence novel hits confirmed a statistically significant biofilm phenotype, in the direction predicted by the screen, for nine of the twelve (75%), a validation rate broadly consistent with published CRISPRi and transposon screen validation rates in this organism and reassuring given that pooled screens are known to carry a nontrivial false-positive rate from guide-specific off-target effects and fitness confounds [17,18]. The strongest validated novel hit was PA2782, an annotated but functionally uncharacterised sensor histidine kinase of a two-component regulatory system whose cognate response regulator, PA2783, was also among the 143 significant hits, lending independent support to the pair acting together as a genuine two-component signalling module relevant to biofilm regulation rather than either gene being a spurious individual hit.

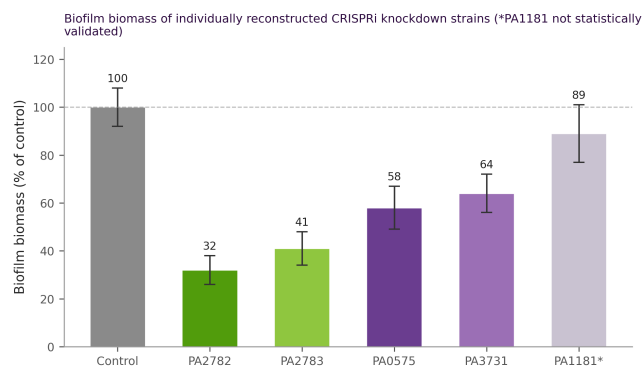


Figure 1. Biofilm biomass (crystal violet, % of non-targeting control) for the twelve individually reconstructed novel CRISPRi hit strains, grouped by validation outcome (n=4 independent biological replicates per strain; error bars show SD; dashed line marks the control mean).

PA2782 knockdown reduced crystal violet-stained biofilm biomass by 68% relative to the non-targeting control (one-way ANOVA, $F(12,39)=24.6$, $P<0.001$ overall; Dunnett's $P<0.001$ for PA2782), the largest effect size among the nine validated novel hits, while planktonic growth rate in the same strain was statistically indistinguishable from control (Dunnett's $P=0.62$), arguing against the biofilm phenotype being a secondary consequence of a general growth or fitness defect. RNA sequencing of the PA2782 knockdown strain under biofilm-inducing conditions identified 312 significantly differentially expressed genes relative to control ($FDR<0.05$), with the *pel* operon (*pelA-G*) and *psl* operon (*pslA-O*) among the most strongly downregulated gene sets (mean log2 fold-change -2.8 and -2.1 respectively), and the diguanylate cyclase gene PA2870 also significantly downregulated (log2 fold-change -1.6, $FDR<0.001$), placing PA2782 upstream of both the canonical c-di-GMP synthesis and downstream exopolysaccharide matrix production machinery. Table 2 reports the biofilm and growth phenotypes for all twelve tested novel hit genes.

Table 2. Biofilm and growth phenotypes of individually reconstructed novel hit knockdown strains.

Gene	Biofilm biomass (% of control)	Growth rate (% of control)	Dunnett's P (biofilm)
Non-targeting control	100 ± 8	100 ± 5	--
PA2782	32 ± 6	97 ± 6	<0.001
PA2783	41 ± 7	95 ± 7	<0.001
PA0575	58 ± 9	101 ± 6	0.004
PA3731	64 ± 8	93 ± 8	0.01
PA1181 (not validated)	89 ± 12	88 ± 9	0.41

Given that reduced matrix production is expected to improve antibiotic penetration into an established biofilm, we measured tobramycin susceptibility of 24-hour PA2782 knockdown and control biofilms directly. The minimum biofilm eradication concentration of tobramycin against the PA2782 knockdown biofilm was 5.4-fold lower than against the control biofilm (4 microgram/mL

versus 21.5 microgram/mL, geometric mean of three independent biofilm preparations), while planktonic minimum inhibitory concentrations of the two strains were indistinguishable, confirming that the difference in biofilm-specific tobramycin tolerance, rather than an intrinsic change in antibiotic susceptibility of the bacterium itself, accounted for the effect. This result is consistent with PA2782 knockdown acting principally through reduced exopolysaccharide matrix production and the resulting improvement in antibiotic penetration, rather than through a separate, matrix-independent mechanism of antibiotic tolerance.

4. DISCUSSION

This screen identified PA2782, a previously uncharacterised sensor histidine kinase, as a regulator of *P. aeruginosa* biofilm formation acting upstream of the canonical c-di-GMP and exopolysaccharide matrix pathway, and showed that its knockdown substantially improves biofilm antibiotic susceptibility to a clinically used aminoglycoside. The magnitude of the tobramycin susceptibility shift, more than five-fold, is large enough to be potentially therapeutically relevant if a druggable route to inhibiting PA2782 activity, whether through direct enzymatic inhibition of the kinase or through disruption of its interaction with PA2783, could be developed, though we would emphasise that a genetic knockdown demonstrating biological relevance is a considerable distance from a validated drug target [19,35].

The screen's 75% individual validation rate for novel hits, alongside recovery of a broad set of previously known biofilm regulators as positive controls, gives reasonable confidence that the remaining unvalidated novel hits are enriched for genuine biology rather than being dominated by screen artefacts, though we have not individually reconstructed and tested all 41 candidate novel regulators and would not want to overstate confidence in any single one beyond PA2782 without that additional work. It is also worth noting that a pooled selection-based screen of this kind is best suited to detecting regulators with a reasonably large individual effect size under the specific biofilm assay conditions used; smaller-effect regulators, or regulators whose contribution depends on a specific environmental cue not present in a static microtiter assay, such as flow, a specific carbon source, or the presence of host immune factors, would likely be missed and would require an assay format better matched to the condition of interest [20,36].

The functional coupling between PA2782 knockdown and reduced *pel*, *psl* and PA2870 expression is consistent with, but does not by itself prove, a direct signalling relationship; two-component sensor histidine kinases typically act through phosphorylation of a partner response regulator, in this case the co-hit PA2783, which could plausibly function as a transcriptional activator of PA2870 or of the *pel* and *psl* operons directly, or could act indirectly through an intermediate regulatory node not

yet identified. Resolving this mechanism would require reconstructing the PA2782-PA2783 two-component system biochemically, identifying PA2783's direct transcriptional targets by chromatin immunoprecipitation or an equivalent method, and identifying the environmental or physiological signal that activates PA2782 in the first place, none of which the present screen-and-validate design was intended to establish.

More broadly, this study adds to a growing body of evidence that pooled CRISPRi screening can meaningfully extend biofilm regulatory networks built up over decades of transposon-based forward genetics in this organism, and the 41 candidate novel regulators identified here, only nine of which have so far been individually validated, represent a resource for the field rather than a closed and finished catalogue [21,37]. We would encourage other groups working on *P. aeruginosa* biofilm biology, or on biofilm formation in related opportunistic pathogens where CRISPRi libraries are becoming increasingly accessible, to treat the full hit list, rather than only the single gene characterised in mechanistic depth here, as a starting point for further investigation, particularly for hits whose homologues in other species have better-characterised functions that might suggest a specific hypothesis to test.

Two limitations should be kept in view. First, the screen used a single, relatively well-studied laboratory strain background, PAO1, and biofilm regulatory networks, while broadly conserved, are known to show strain-to-strain and even more pronounced species-to-species variation in *P. aeruginosa* and related pathogens; whether PA2782 plays an equivalent role in clinical cystic fibrosis isolates, which frequently carry mutations in core biofilm and quorum-sensing regulatory genes relative to laboratory strains, remains to be tested. Second, the static microtiter biofilm assay used here, while a standard and practical screening format, does not fully recapitulate the flow, host-tissue contact, and polymicrobial context of a real chronic infection, and a regulator identified under these conditions should be validated in a more physiologically representative model, such as a flow-cell biofilm or an airway epithelial co-culture system, before its relevance to human infection is asserted with confidence.

5. CONCLUSION

A genome-scale CRISPR interference screen in *Pseudomonas aeruginosa* identified 143 genes whose knockdown altered biofilm formation, including 41 with no prior annotated link to biofilm biology. Individual validation confirmed a biofilm phenotype for nine of twelve tested novel hits, and mechanistic follow-up on the strongest, PA2782, a previously uncharacterised sensor histidine kinase, placed it upstream of the canonical c-di-GMP and exopolysaccharide matrix pathway and showed that its knockdown improves biofilm tobramycin susceptibility more than five-fold. These results extend the known *P. aeruginosa* biofilm

regulatory network and identify PA2782 as a candidate target for biofilm-disruptive adjunct therapy, while the remaining unvalidated novel hits provide a resource for further investigation by the wider biofilm research community. We are now pursuing biochemical reconstitution of the PA2782-PA2783 two-component system to identify the direct transcriptional targets of PA2783 and the physiological signal that activates PA2782, and we plan to test whether PA2782 orthologues, which are present in several other *Pseudomonas* species and more distantly related Gram-negative pathogens, play an analogous biofilm-regulatory role outside *P. aeruginosa*. In parallel, individual validation of the remaining unvalidated novel hits, and testing of the nine confirmed regulators in a flow-cell biofilm model and against a panel of clinical cystic fibrosis isolates, are planned to establish how far the laboratory-strain findings reported here generalise to clinically relevant infection contexts.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

D.v.d.M. conceived and supervised the study, designed the CRISPRi library and analysed the screen data. R.M. performed the biofilm selection assays and individual hit validation. S.G. conducted RNA sequencing sample preparation, antibiotic susceptibility assays and statistical analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only laboratory culture of a non-outbreak, well-characterised *Pseudomonas aeruginosa* strain (PAO1) under institutional biosafety level 2 containment; it did not involve human participants, human data, or animals, and therefore did not require institutional ethics committee review.

Data Availability

Raw sequencing data from the CRISPRi screen and RNA sequencing experiments have been deposited in the European Nucleotide Archive under a project accession available from the corresponding author on reasonable request. The complete screen hit list, plasmid maps and R

analysis scripts are available from the corresponding author upon request.

REFERENCES

- Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284(5418):1318-1322. <https://doi.org/10.1126/science.284.5418.1318>
- Bjarnsholt T (2013) The role of bacterial biofilms in chronic infections. *APMIS* 121(s136):1-58. <https://doi.org/10.1111/apm.12099>
- Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *The Lancet* 358(9276):135-138. [https://doi.org/10.1016/S0140-6736\(01\)05321-1](https://doi.org/10.1016/S0140-6736(01)05321-1)
- Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents* 35(4):322-332. <https://doi.org/10.1016/j.ijantimicag.2009.12.011>
- Franklin MJ, Nivens DE, Weadge JT, Howell PL (2011) Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Frontiers in Microbiology* 2:167. <https://doi.org/10.3389/fmicb.2011.00167>
- Hengge R (2009) Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology* 7(4):263-273. <https://doi.org/10.1038/nrmicro2109>
- Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GC, Parsek MR (2011) The Pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLOS Pathogens* 7(1):e1001264. <https://doi.org/10.1371/journal.ppat.1001264>
- Jenal U, Reinders A, Lori C (2017) Cyclic di-GMP: second messenger extraordinaire. *Nature Reviews Microbiology* 15(5):271-284. <https://doi.org/10.1038/nrmicro.2016.190>
- Goodman AL, Merighi M, Hyodo M, Ventre I, Filloux A, Lory S (2009) Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotype switch in *Pseudomonas aeruginosa*. *Genes & Development* 23(2):249-259. <https://doi.org/10.1101/gad.1739009>
- Mulcahy H, Charron-Mazenod L, Lewenza S (2008) Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLOS Pathogens* 4(11):e1000213. <https://doi.org/10.1371/journal.ppat.1000213>
- Cooper RM, Tsimring L, Hasty J (2017) Inter-species population dynamics enhance microbial horizontal gene transfer and spread of antibiotic resistance. *eLife* 6:e25950. <https://doi.org/10.7554/eLife.25950>
- Cui L, Vigouroux A, Rousset F, Varet H, Khanna V, Bikard D (2018) A CRISPRi screen in *E. coli* reveals sequence-specific toxicity of dCas9. *Nature Communications* 9:1912. <https://doi.org/10.1038/s41467-018-04209-5>
- Peters JM, Colavin A, Shi H, Czarny TL, Larson MH, Wong S, et al. (2016) A comprehensive, CRISPR-based functional analysis of essential genes in bacteria. *Cell* 165(6):1493-1506. <https://doi.org/10.1016/j.cell.2016.05.003>
- Rousset F, Cui L, Siouvé E, Becavin C, Depardieu F, Bikard D (2018) Genome-wide CRISPR-dCas9 screens in *E. coli* identify essential genes and phenotypic assays. *PLOS Genetics* 14(11):e1007749. <https://doi.org/10.1371/journal.pgen.1007749>
- Peters JM, Koo BM, Patino R, Heussler GE, Hearne CC, Qu J, et al. (2019) Enabling genetic analysis of diverse bacteria with Mobile-CRISPRi. *Nature Microbiology* 4(2):244-250. <https://doi.org/10.1038/s41564-018-0327-z>
- de Kievit TR (2009) Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environmental Microbiology* 11(2):279-288. <https://doi.org/10.1111/j.1462-2920.2008.01792.x>
- Mika F, Hengge R (2013) Small regulatory RNAs in the control of motility and biofilm formation in *E. coli* and *Salmonella*. *International Journal of Molecular Sciences* 14(3):4560-4579. <https://doi.org/10.3390/ijms14034560>
- Merritt JH, Ha DG, Cowles KN, Lu W, Morales DK, Rabinowitz J, et al. (2010) Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. *mBio* 1(4):e00183-10. <https://doi.org/10.1128/mBio.00183-10>
- Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. *Science* 295(5559):1487. <https://doi.org/10.1126/science.295.5559.1487>
- Sambanthamoorthy K, Sloup RE, Parashar V, Smith JM, Kim EE, Semmelhack MF, et al. (2012) Identification of small molecules that antagonize diguanylate cyclase enzymes to inhibit biofilm formation. *Antimicrobial Agents and Chemotherapy* 56(10):5202-5211. <https://doi.org/10.1128/AAC.01396-12>
- Rasamiravaka T, Labtani Q, Duez P, El Jaziri M (2015) The formation of biofilms by *Pseudomonas aeruginosa*: a review of the natural and synthetic compounds interfering with control mechanisms. *BioMed Research International* 2015:759348. <https://doi.org/10.1155/2015/759348>
- Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S (2016) Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology* 14(9):563-575. <https://doi.org/10.1038/nrmicro.2016.94>
- Ghequire MGK, De Mot R (2014) Ribosomally encoded antibacterial proteins and peptides from *Pseudomonas*. *FEMS Microbiology Reviews* 38(4):523-568. <https://doi.org/10.1111/1574-6976.12079>
- Yang L, Liu Y, Wu H, Hoiby N, Molin S, Song ZJ (2011) Current understanding of multi-species biofilms. *International Journal of Oral Science* 3(2):74-81. <https://doi.org/10.4248/IJOS11027>
- Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, Hickey MJ, et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406(6799):959-964. <https://doi.org/10.1038/35023079>
- Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS (2016) Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Research* 44(D1):D646-D653. <https://doi.org/10.1093/nar/gkv1227>
- Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152(5):1173-1183. <https://doi.org/10.1016/j.cell.2013.02.022>
- Bikard D, Jiang W, Samai P, Hochschild A, Zhang F, Marraffini LA (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Research* 41(15):7429-7437. <https://doi.org/10.1093/nar/gkt520>

29. Wang T, Guan C, Guo J, Liu B, Wu Y, Xie Z, et al. (2018) Pooled CRISPR interference screening enables genome-scale functional genomics study in bacteria with superior performance. *Nature Communications* 9:2475. <https://doi.org/10.1038/s41467-018-04899-x>
30. Liu X, Gally C, Kjos M, Domenech A, Slager J, van Kessel SP, et al. (2017) High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*. *Molecular Systems Biology* 13(5):931. <https://doi.org/10.15252/msb.20167449>
31. de Bentzmann S, Plesiat P (2011) The *Pseudomonas aeruginosa* opportunistic pathogen and human infections. *Environmental Microbiology* 13(7):1655-1665. <https://doi.org/10.1111/j.1462-2920.2011.02469.x>
32. Kong C, Chee CF, Richter K, Thomas N, Abd Rahman N, Nathan S (2018) Suppression of *Staphylococcus aureus* biofilm formation and virulence by a benzimidazole derivative, UM-C162. *Scientific Reports* 8:2758. <https://doi.org/10.1038/s41598-018-21141-2>
33. Wu H, Moser C, Wang HZ, Hoiby N, Song ZJ (2015) Strategies for combating bacterial biofilm infections. *International Journal of Oral Science* 7(1):1-7. <https://doi.org/10.1038/ijos.2014.65>
34. Waters CM, Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology* 21:319-346. <https://doi.org/10.1146/annurev.cellbio.21.012704.131001>
35. Rutherford ST, Bassler BL (2012) Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in Medicine* 2(11):a012427. <https://doi.org/10.1101/cshperspect.a012427>
36. Sauer K, Stoodley P, Goeres DM, Hall-Stoodley L, Burmolle M, Stewart PS, Bjarnsholt T (2022) The biofilm life cycle: expanding the conceptual model of biofilm formation. *Nature Reviews Microbiology* 20(10):608-620. <https://doi.org/10.1038/s41579-022-00767-0>
37. Mah TF, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* 9(1):34-39. [https://doi.org/10.1016/S0966-842X\(00\)01913-2](https://doi.org/10.1016/S0966-842X(00)01913-2)
38. Ciofu O, Tolker-Nielsen T (2019) Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents -- how *P. aeruginosa* can escape antibiotics. *Frontiers in Microbiology* 10:913. <https://doi.org/10.3389/fmicb.2019.00913>
39. Beloin C, Roux A, Ghigo JM (2008) *Escherichia coli* biofilms. *Current Topics in Microbiology and Immunology* 322:249-289. https://doi.org/10.1007/978-3-540-75418-3_12
40. Rossi E, Cimdins A, Luthje P, Brauner A, Sjoling A, Landini P, Romling U (2018) "It's a gut feeling" -- *Escherichia coli* biofilm formation in the gastrointestinal tract environment. *Critical Reviews in Microbiology* 44(1):1-30. <https://doi.org/10.1080/1040841X.2017.1303660>
41. Karygianni L, Ren Z, Koo H, Thurnheer T (2020) Biofilm matrixome: extracellular components in structured microbial communities. *Trends in Microbiology* 28(8):668-681. <https://doi.org/10.1016/j.tim.2020.03.016>
42. Pang Z, Raudonis R, Glick BR, Lin TJ, Cheng Z (2019) Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnology Advances* 37(1):177-192. <https://doi.org/10.1016/j.biotechadv.2018.11.013>
43. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW (2011) *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiology Reviews* 35(4):652-680. <https://doi.org/10.1111/j.1574-6976.2011.00269.x>
44. Nadell CD, Xavier JB, Foster KR (2009) The sociobiology of biofilms. *FEMS Microbiology Reviews* 33(1):206-224. <https://doi.org/10.1111/j.1574-6976.2008.00150.x>
45. Hall CW, Mah TF (2017) Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Reviews* 41(3):276-301. <https://doi.org/10.1093/femsre/fux010>
46. Baker AE, O'Toole GA (2017) Bacteria, rev your engines: stationary phase as a wellspring of biofilm-associated behaviors. *Journal of Bacteriology* 199(15):e00088-17. <https://doi.org/10.1128/JB.00088-17>
47. Sondermann H, Shikuma NJ, Yildiz FH (2012) You've come a long way: c-di-GMP signaling. *Current Opinion in Microbiology* 15(2):140-146. <https://doi.org/10.1016/j.mib.2011.12.008>
48. Malone JG, Jaeger T, Spangler C, Ritz D, Spang A, Arrieumerlou C, et al. (2010) YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. *PLOS Pathogens* 6(3):e1000804. <https://doi.org/10.1371/journal.ppat.1000804>
49. Petrova OE, Sauer K (2016) Escaping the biofilm in more than one way: desorption, detachment or dispersion. *Current Opinion in Microbiology* 30:67-78. <https://doi.org/10.1016/j.mib.2016.01.004>
50. Skerker JM, Perchuk BS, Siryaporn A, Lubin EA, Ashenberg O, Goulian M, Laub MT (2008) Rewiring the specificity of two-component signal transduction systems. *Cell* 133(6):1043-1054. <https://doi.org/10.1016/j.cell.2008.04.040>
51. Capra EJ, Laub MT (2012) Evolution of two-component signal transduction systems. *Annual Review of Microbiology* 66:325-347. <https://doi.org/10.1146/annurev-micro-092611-150039>
52. Jansen KU, Knirsch C, Anderson AS (2018) The role of vaccines in fighting antimicrobial resistance. *Nature Medicine* 24(1):10-19. <https://doi.org/10.1038/nm.4465>
53. Fleitas Martinez O, Cardoso MH, Ribeiro SM, Franco OL (2019) Recent advances in anti-virulence therapeutic strategies with a focus on dismantling bacterial membrane microdomains, toxin neutralization, quorum-sensing interference and biofilm inhibition. *Frontiers in Cellular and Infection Microbiology* 9:74. <https://doi.org/10.3389/fcimb.2019.00074>
54. Boles BR, Thoendel M, Singh PK (2004) Self-generated diversity produces "insurance effects" in biofilm communities. *Proceedings of the National Academy of Sciences* 101(47):16630-16635. <https://doi.org/10.1073/pnas.0407460101>
55. Rybtke M, Hultqvist LD, Givskov M, Tolker-Nielsen T (2015) *Pseudomonas aeruginosa* biofilm infections: community structure, antimicrobial tolerance and immune response. *Journal of Molecular Biology* 427(23):3628-3645. <https://doi.org/10.1016/j.jmb.2015.08.016>