MICROBIAL INFECTIONS AND VIRULEN

Francesco Imperi – Arianna Tavanti

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Pisa4: a potential candidate for *Mycobacterium tuberculosis* treatment

A. Fusco[a], A. Bonacorsi[a], CC. Ko[c], M. J. Lauer[c], L. Rindi[d], A. Tavanti[a], A.H. Diacon[b], G. F. Hatfull[d], S. Janssen[b*], M. Di Luca[a,e*]

[a] Department of Biology, University of Pisa, Pisa, Italy

[b] TASK, Cape Town, South Africa

[c] Department of Biological Sciences, University of Pittsburgh, Pittsburgh, United States

[d] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy.

[e] Antimicrobial Resistance Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy

*These authors share the last authorship.

Pisa4 is a recently identified cluster K1 mycobacteriophage that lacks integrase and encodes a partial immunity repressor. We aimed to evaluate its antibacterial activity against *Mycobacterium tuberculosis* (MTB) following complete deletion of the repressor gene, to prevent reversion to a temperate phenotype, while also exploring its host range in *Mycobacterium abscessus* (MAB) another clinically relevant species. Pisa4 was engineered using BRED, generating the Pisa4Δ_IR mutant, which was tested in time-kill assays against MTB H37Rv. Sera from TB patients in Cape Town were used to assess phage neutralization, and their MTB isolates were evaluated for susceptibility. In parallel, host range was further explored in 48 clinical isolates of MAB, collected in Pisa, Rome and Palermo. The repressor gene of Pisa4 was successfully deleted. Pisa4Δ_IR infected MTB H37Rv, showing an increase in plaque-forming units (~3.5 $\log_{10}$). From day 5 post-infection, it suppressed bacterial growth, maintaining colony-forming units close to baseline, while untreated controls increased (~2 $\log_{10}$). Overall, 30/34 (88%) clinical MTB isolates were susceptible with an EOP ≥0.01. In contrast, a limited activity was revealed against MAB, with lytic infection observed in only 8/48 isolates. No serum neutralization was detected. Deletion of the partial repressor ensures stable lytic activity. Pisa4Δ_IR effectively controls MTB growth *in vitro* and shows a broad host range among the MTB isolates, while displaying limited activity against MAB. These findings support its potential as a targeted anti-MTB therapeutic and warrant further evaluation across genetically diverse isolates, as well as in intracellular and low-metabolic conditions.

DksA–(p)ppGpp interplay is a key determinant of *Pseudomonas aeruginosa* extracellular and intracellular infection

Alessandra Guiducci[1], Alessandra Fortuna[1], Serena Ciolfi[1], Marta Mellini[1], Andrea Sabatini[2], Flavia Giannessi[1], Elisabetta Affabris[1], Heike Bähre[3], Francesco Imperi[1,4,5], Paolo Visca[1,4,5], Giordano Rampioni[1,4], Alessandra Sacchi[1], Livia Leoni[1]

[1]*Department of Science, University Roma Tre, Rome, Italy;* [2]*Immunology Research Area, Innate Lymphoid Cells Unit, IRCCS Bambino Gesù Children's Hospital, Rome, Italy;* [3]*Research Core Unit Metabolomics, Hannover Medical School, Hannover, Germany;* [4]*IRCCS Fondazione Santa Lucia, Rome, Italy;* [5]*NBFC, National Biodiversity Future Center, Palermo, Italy*

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen responsible for severe acute and chronic infections. Although traditionally considered extracellular, it can persist within host cells, contributing to immune evasion and antibiotic resistance. Despite its clinical relevance, the molecular mechanisms enabling intracellular survival remain poorly understood.

The stringent response (SR), mediated by the alarmone (p)ppGpp and the transcriptional regulator DksA, is a key global regulatory system controlling bacterial adaptation and virulence. Here, the interplay between (p)ppGpp and DksA in regulating virulence and intracellular persistence of *P. aeruginosa* was investigated. Using a reverse genetics approach, mutants lacking DksA proteins (Δ*dksA*), (p)ppGpp (Δ*rs*, lacking the *relA* and *spoT* genes), or both (Δ*dksA-rs*) were generated. All SR-mutant strains showed reduced motility, impaired production of virulence factors, and attenuated pathogenicity in *Galleria mellonella*.

Importantly, SR mutants displayed decreased cytotoxicity and impaired intracellular survival in human lung epithelial cells compared to the wild type, with the Δ*dksA-rs* showing the strongest defect. This phenotype was correlated with reduced expression of the Type III Secretion System effector ExoS and an impaired ability to evade intracellular degradative pathways. Confocal microscopy revealed increased colocalization of the Δ*dksA-rs* mutant with LAMP1-positive compartments, indicating impaired escape from lysosomal degradation. Consistently, inhibition of lysosomal acidification and autophagosome-lysosome fusion by bafilomycin restored mutant survival.

Overall, (p)ppGpp and DksA act both additively to promote *P. aeruginosa* virulence and intracellular persistence, highlighting the SR as a critical determinant of host-pathogen interaction and a promising target for the development of novel antimicrobial strategies.

Targeting *de novo* L-cysteine biosynthesis (DeNoCB) in *Pseudomonas aeruginosa* for novel antimicrobial strategies

Rebecca Martedì[a], Sarah Hijazi[a], Jole Maria Lucia D'Angelo[b], Francesco Guggino[c], Marialaura Marchetti[d], Matilda Ymeraj[a], Gian Marco Elisi[a], Giovanni Bottegoni[a], Giannamaria Annunziato[b], Marco Pieroni[b], Gabriele Costantino[b], Stefano Bettati[c, d], Barbara Campanini[b], Emanuela Frangipani[a]

[a] *Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy*
[b] *Department of Food and Drug, University of Parma, Parma, Italy*
[c] *Interdepartmental center Biopharmanet-TEC, University of Parma, Parma, Italy*
[d] *Department of Medicine and Surgery, University of Parma, Parma, Italy*

Bacterial DeNoCB is a key metabolic hub and a promising antibiotic target due to its absence in humans, yet it remains poorly explored in *P. aeruginosa*[1,2]. Four key DeNoCB enzymes - CysK (PA2709), CysM (PA0932), CysE (PA3816), and CysH (PA1756) - were identified and physiologically/biochemically characterized. Their roles were investigated using deletion mutants grown in minimal media with different S-sources (sulfate, thiosulfate, or L-cysteine). Single deletions of *cysM* or *cysK* did not result in cysteine auxotrophy, whereas the double Δ*cysM*Δ*cysK* mutant showed no growth for up to 18 h, followed by recovery at later time points, suggesting the presence of alternative pathway(s)[3]. CysE was found to be essential for DeNoCB, by supplying the key intermediate *O*-acetylserine, as the Δ*cysE* mutant failed to grow on both sulfate and thiosulfate. Finally, the Δ*cysH* mutant was confirmed to be a cysteine auxotroph, but only when growing on sulfate.

Notably, both Δ*cysH* and Δ*cysE* exhibited reduced virulence in the *Galleria mellonella* infection model compared to the wild-type strain, thus emerging as promising antimicrobial targets.

Recombinant production and functional characterization of all four enzymes enabled compound library screening and inhibitor validation, both *in vitro* and in bacterial cultures. Preliminary results identified hit compounds targeting CysE (IC_{50} = ~30 µM, >60% growth inhibition) and CysH (IC_{50} = ~50 µM, >30% growth inhibition), while assays on CysK and CysM are ongoing.

Overall, these findings provide new insights into *P. aeruginosa* metabolic and regulatory networks, highlighting the key role of sulfur metabolism in bacterial physiology and pathogenicity.

References

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Drug repurposing to inhibit *Pseudomonas aeruginosa* adaptation to the cystic fibrosis lung environment

Claudia Ridolfi[1], Marta Mellini[1], Lavinia Renzi[1], Valeria Stornelli[1], Paolo Visca[1,2,3], Francesco Imperi[1,2,3], Livia Leoni[1], Giordano Rampioni[1,2]

[1]Department of Science, University Roma Tre, Rome, Italy; [2]IRCCS Fondazione Santa Lucia, Rome, Italy; [3]NBFC, National Biodiversity Future Center, Palermo, Italy

The advent of CFTR modulators has led to remarkable improvements in lung function and overall health in many people with cystic fibrosis (CF). However, these therapies do not eradicate chronic lung infections caused by *Pseudomonas aeruginosa*, which remain a major health threat for people with CF.

Traditional antimicrobial discovery mainly relies on screening compounds in nutrient-rich media that poorly reflect *in vivo* conditions and rarely yields novel therapeutic options. Adapting screening conditions to better mimic the host environment has emerged as a promising strategy to uncover previously unrecognized antimicrobial activities in existing drugs. In particular, repurposed drugs that inhibit *P. aeruginosa* growth or biofilm formation in the synthetic cystic fibrosis medium (SCFM), which closely reproduces the chemical composition of CF sputum, may reduce bacterial burden and pathogenicity in the CF lung.

On this basis, over 3,000 FDA-approved drugs were screened in parallel in SCFM and in a standard rich medium. This approach allowed identifying drugs with potent and previously unrecognized antimicrobial or antibiofilm activity against *P. aeruginosa* specifically in SCFM. The best hits showed robust *in vitro* activity against the reference strain PAO1 and a panel of clinical CF isolates. Ongoing studies aim to identify their specific molecular target(s) and clarify their mechanism of action.

By leveraging drug repurposing in a physiologically relevant context, this study uncovered the antimicrobial potential of safe drugs that could improve the treatment of *P. aeruginosa* lung infections in people with CF.

Two AraC/XylS-family transcription factors contribute to virulence and oxidative stress tolerance in *Acinetobacter baumannii* AB5075

Tommaso Tacchetti, Gianni Prosseda

Department of Biology and Biotechnology "Charles Darwin", Sapienza University of Rome, Italy

Acinetobacter baumannii is a major cause of hospital-acquired infection and a critical antimicrobial resistance threat. As treatment options narrow, targeting virulence regulators may provide an alternative therapeutic strategy. We focused on on AraC/xylS-like transcriptional regulators (AFTR) in *A. baumannii* 5075 (AB5075), *virF* and *alkR.* VirF from AB5075 is conserved across diverse recent clinical isolates and shares predicted structural similarity with VirF of *Shigella flexneri,* while previous work has implicated *alkR* in *Galleria mellonella* infection. To test their contribution to virulence, we performed *G. mellonella* killing assay and found that disruption of *virF* or *alkR* significantly attenuated virulence in vivo. We then assessed antibiotic susceptibility, biofilm formation and architecture, motility and growth under multiple stress conditions. Although the mutants did not differ from the wild type in antibiotic susceptibility, biofilm, or motility, both showed impaired growth under paraquat stress, but not hydrogen peroxide stress. These findings suggest that virFA and alkR contribute to virulence through adaptation to oxidative stress, potentially in response to superoxide-generating conditions. One next step will be to test whether these regulators control oxidative-stress genes and intracellular survival in THP-1 cells. Together, these findings identify VirFA and AlkR as candidate virulence regulators in *A. baumannii* AB5075, and, given the reported inhibition of AraC/XylS-family regulators by fatty acids, suggest a potential route for anti-virulence intervention.

Rhizosphere microbiome engineering and root exudate metabolites have the potential to enhance the bioremediation of petroleum hydrocarbons

Gabriele Capriglia[1], Francesca Mapelli[1], Eleonora Rolli[1], Lorenzo Vergani[1], Luigi Bazzana[1], Elisa Ghitti[1], Sara Borin[1]

[1] Department of Food, Environmental and Nutritional Sciences - DeFENS, University of Milan, Italy

Nature-based solutions for historically polluted sites remediation include the enhancement of the degradative potential selected in the soil microbiome over time. Biostimulation of degradative microorganisms can be achieved by adding specific compounds directly to the soil or it can be mediated by plants, that act synergistically with the soil microbiome in rhizoremediation approaches, often prompted by the addition of selected microorganisms.
Here, sunflower was employed alone or supplemented by degradative strains to optimize petroleum hydrocarbon (PHC) remediation of an industrial polluted soil. The dynamics of the rhizosphere microbiome and the abundance of PHC catabolic genes were monitored on the soil metagenome by metataxonomic and qPCR analyses, while the efficacy of microbial inoculation in boosting PHC removal was chemically assessed. Moreover, leveraging untargeted metabolomics data, the role of specific root exudate metabolites as possible biostimulants of PHC degradation was evaluated *in vitro* providing them to the microbial cells detached from the target soil.
In soil, we observed that sunflower recruited a rhizosphere community diverse from that evolving over time in unplanted microcosms. Moreover, bioaugmentation with degrading strains further steered the structure of the overall rhizosphere microbiome, increasing the abundance of catabolic genes and the removal of PCHs compared to non-inoculated plants. *In vitro*, soil-detached microbiome showed improved PHC degradation when supplemented by the tested metabolites, suggesting that these plant-derived molecules trigger degradation pathways of the native bacterial populations naturally selected in the historical contaminated soil.

Our findings demonstrate that the understanding of ecological interactions in the rhizosphere, mediated by invading degrading strains and by plant via root exudation is essential for optimizing the design of rhizoremediation interventions.

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