Elucidating key steps of *Pseudomonas aeruginosa* infection by DEV lytic phage for phage therapy

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Background

Pseudomonas aeruginosa (*Pa)*, a Gram-negative opportunistic bacteria, is the leading cause of nosocomial and chronic respiratory infections in people with cystic fibrosis (CF). *Pa* infections are frequently resistant to antibiotics. Phage therapy, leveraging the life cycle of lytic phages (viruses that kill bacteria), shows significant potential as a treatment for bacterial infections resistant to antibiotics.

A cocktail composed by four phages (CK4), able to kill *Pa* clinical strains isolated from people with CF, was recently developed[1]. CK4 resistant mutants were isolated. All presented mutations in *wzy,* which is involved in the biosynthesis of the lipopolysaccharide (LPS) component of the outer membrane (OM)[2] **(Fig. 1).**

A CK4 component, i.e. the *Schitoviridae* **DEV** phage, can infect both the wt smooth strain producing long LPS with the O-antigen (**Fig. 1**, c), or rough strains producing short LPS variants with a truncated core (i.e.*algC, galU* and *wapH* mutants; tc in **Fig.1**), whereas strains producing LPS containing a single O-antigen repeat (i.e. *wzy* mutants; u+1 in **Fig. 1**) are DEV resistant.

DEV gp53 gene encodes long tail fibers that are usually the receptor binding proteins of phages. A Δgp53 DEV mutant was constructed using CRISPR-Cas editing**.** The mutant phage can infect rough mutants, but cannot grow in PAO1 or *wzy* strains **(Fig. 2)[3].**

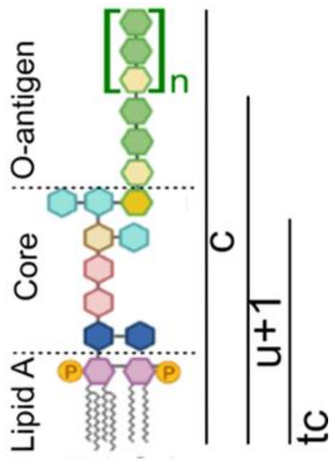

Figure 1. Predicted LPS structure in phage resistant mutants.

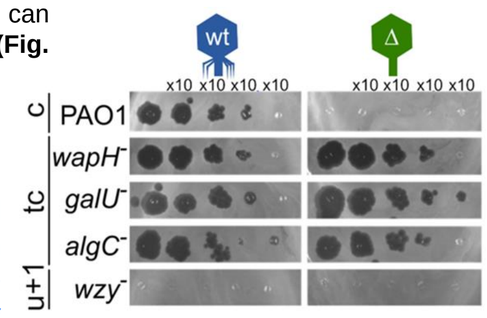

Figure 2. Replica Plating on *Pa* Mutants of DEV (wt) and Δgp53 (Δ)

Hypothesis I

DEV may enjoy a two-receptor adsorption mechanism. The first receptor could be the O-antigen recognized by Gp53. The second receptor could be exposed in rough mutants, making the interaction with the O-antigen dispensable, and hidden, or absent, in *wzy* mutants.

Results I

1. Isolation of DEV Resistant Double Mutants and complementation of the phage resistant phenotype.

Two spontaneous DEV resistant mutants of the phage-sensitive *galU* strain were isolated, carrying an extra mutation besides that in *galU*, namely:

- ✓ ***galU lptD*:** it contains a 33 bp long deletion in *lptD,* encoding the LPS OM transporter LptD.
- ✓ ***galU rpoH:*** it contains a missense mutation in *rpoH* encoding σ^H sigma factor.

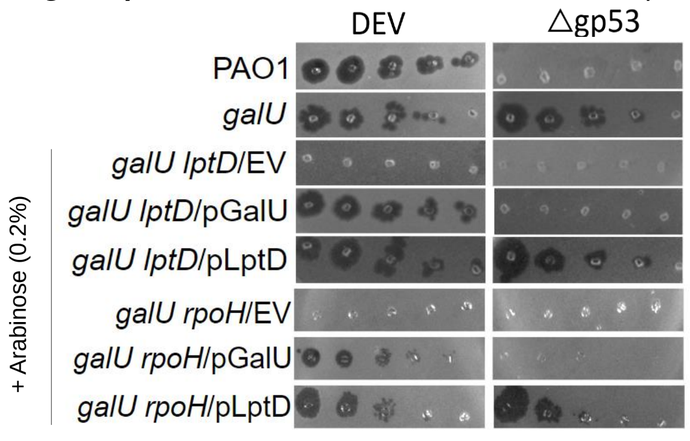

Figure 3. Complementation assay of phage resistant mutants. Our working hypothesis on adsorption: *galU lptD*|EV: Empty vector, *galU* and *lptD* mutations are present, no receptors available. *galU lptD*|pGalU: DEV uses the O-antigen as receptor. △gp53 lacks tail fibers to attach to O-antigen. *galU lptD*|pLptD: DEV and Δgp53 use LptD as receptor.

In *galU lptD*:

- When *lptD* is complemented, adsorption of both phages is restored.
- When *galU* is complemented, only DEV adsorption is restored.

The *lptD* mutation does not prevent phage growth if a smooth LPS can be produced and the phage has Gp53 tail fibers.

In *rpoH lptD*:

- When *galU* is complemented, only DEV adsorption is restored.
- *lptD* overexpression suppresses the *rpoH* mutation.

The *rpoH* mutation interferes with adsorption, most likely by preventing the production or export to the OM of the second receptor.

2. DEV and Δp53 Adsorption tests

To assess if adsorption is the defective step in resistant mutants

To assess if adsorption is restored in presence of complementing plasmids

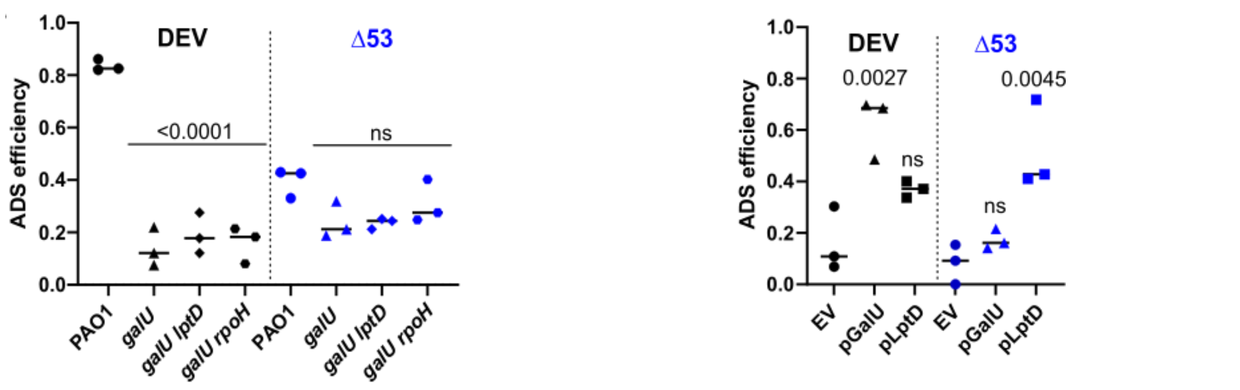

Figure 4. Adsorption efficiency of DEV and Δgp53 to the indicated mutants (left) or to *galU lptD* double mutant carrying the indicated plasmids. EV, empty vector, pGM931. The lines represents average. P calculated with One-way ANOVA and Šídák's multiple comparisons test is reported.

- The absence of O-antigen prevents stable adsorption by DEV.
- Consistently, Δp53 always has inefficient adsorption increased when *lptD* is overexpressed.

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Hypothesis II

The LptD protein is DEV secondary receptor. Alternatively, another LPS form not produced in the *lptD galU* mutant could be used by the phage as receptor.

Results II

3. Adsorption to a *Pa* strain with LPS depletion

To evaluate if LPS is required for adsorption

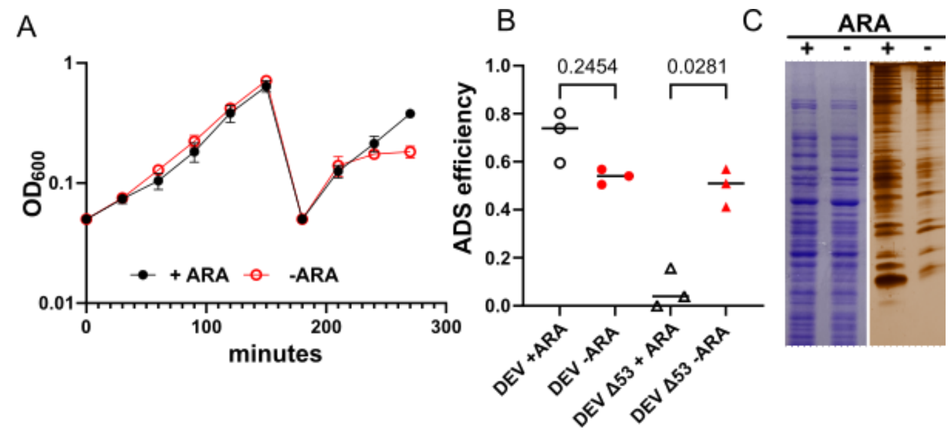

Figure 5. LPS depletion stimulates DEV Δ53 adsorption. A. Growth of the *araBp-lpxA* strain in presence or absence of 0.2% arabinose. The adsorption assay (B) and LPS analysis (C) were executed on bacteria collected at the last time point. B. Adsorption efficiency. Significance estimated with One-way ANOVA and Šídák's multiple comparisons test. C. LPS (right panel) and proteins (left panel).

- LpxA catalyzes one of the first steps of LPS biosynthesis.
- DEV can adsorb to LpxA-depleted cells in spite of severe LPS depletion.
- Δp53 adsorption improved significantly upon LpxA (and LPS) depletion.

4. Analysis of LPS prepared from strains defective in LPS production or transport

To evaluate the effects of mutations on LPS pattern

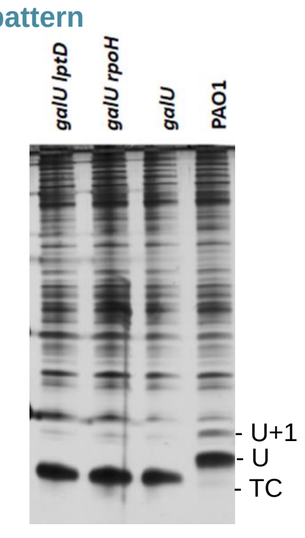

Figure 5. LPS migration on 18% tricin gel and silver staining.

galU lptD and *galU rpoH* double mutants show an LPS pattern almost identical to that of the *galU* single mutant.

5. Adsorption of DEV to a rough *E. coli* strain expressing *Pa lptD*

To assess whether LptD promote adsorption in a heterologous system

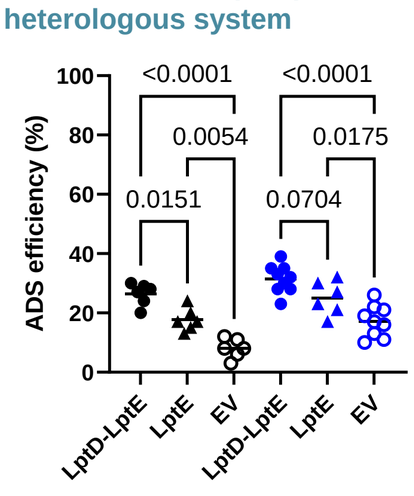

Figure 6. Adsorption of DEV (black) and Δgp53 (blue) to *E. coli* JW3606 (Δ*rfaG*) expressing the indicated Pa proteins. EV, empty vector. Significance estimated with One-way ANOVA and Šídák's multiple comparisons test.

LptE forms a complex with LptD and is essential for the correct assembly of LptD on the OM. When both *Pa lptD* and *lptE* genes are expressed, the adsorption of DEV and Δgp53 to *E. coli* slightly but consistently increases**.**

Conclusions

- DEV can adsorb either to the O-antigen with the Gp53 long tail fibers or to the secondary receptor with another unidentified receptor binding protein.
- DEV Δgp53 cannot bind the O-antigen. However it can bind the secondary receptor when not hindered by LPS capped with a single or multiple O-antigen repeats.
- Adsorption to the secondary receptor only is hardly detectable in conventional adsorption tests suggesting that it can be unstable.
- Data supports the essential LptD-LptE complex as the secondary receptor of DEV phage.

Bibliography

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3. Cingolani G, Lokareddy R, Hou CF, Forti F, Iglesias S, Li F, Pavlenok M, Niederweis M, Briani F. Integrative structural analysis of *Pseudomonas* phage DEV reveals a genome ejection motor. Res Sq [Preprint]. 2024 doi: 10.21203/rs.3.rs-3941185/v1.