Characterization of bacterial resistance to bacteriophages targeting *Escherichia coli* strains causing bovine mastitis

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Introduction: Bacterial phage resistance is a major challenge in phage therapy because it impairs the treatment efficacy and alters the bacterial fitness. A recent study highlighted that bacteria isolated from bovine mastitis became resistant to bacteriophages after 5 h of incubation in raw milk. The aims of this study were to evaluate the *in vitro* onset of resistance in raw milk and to characterize these bacterial mutants. To assess the reversibility of resistant bacteria, phage adaptation was performed to restore phage efficacy.

Isolation and confirmation of resistant mutants

Materials and methods (MM):

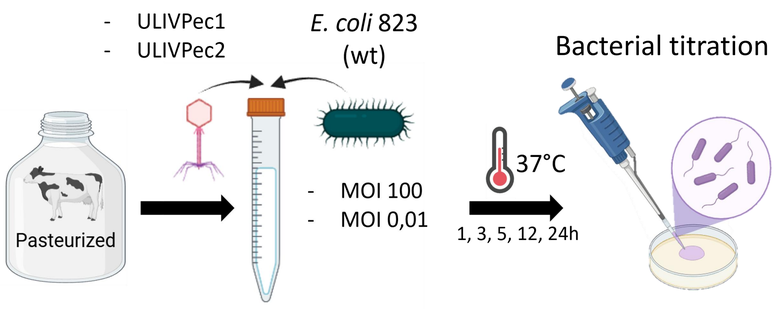

Figure 1: Isolation of mutants in pasteurized milk.

In the case of bacterial regrowth (**Figure 2**), colonies were collected. Confirmation of the resistance of these colonies was evaluated with the spot test method, which consisted in a 4 µl phage spot on a bacterial overlay (OD_{600}=0,3) of each bacterial strain. After overnight incubation, the lysis was characterized as confluent, opaque or no lysis.

Results (Res): Seven strains were isolated from milk samples, of which 6/7 strains were resistant to both phages (**Table I**).

Stability of resistance

MM:

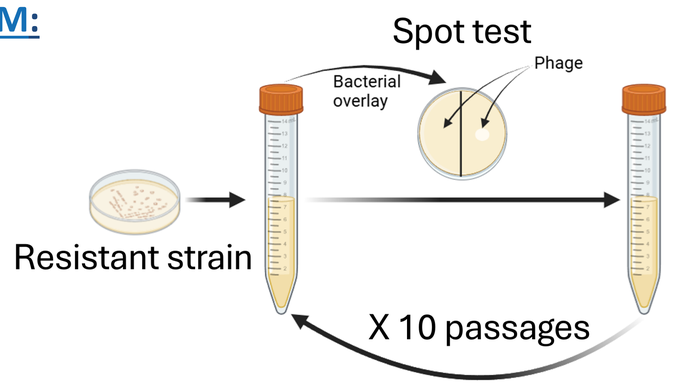

Figure 3: Mutants were serially cultured in LB broth.

Res: After 10 passages, strains m823_D, m823_G and m823_J lost their resistance and were identified as m823_Dp, m823_Gp and m823_Jp. m823_Np Was used in subsequent assays although it didn't lose its resistance (**Table I**).

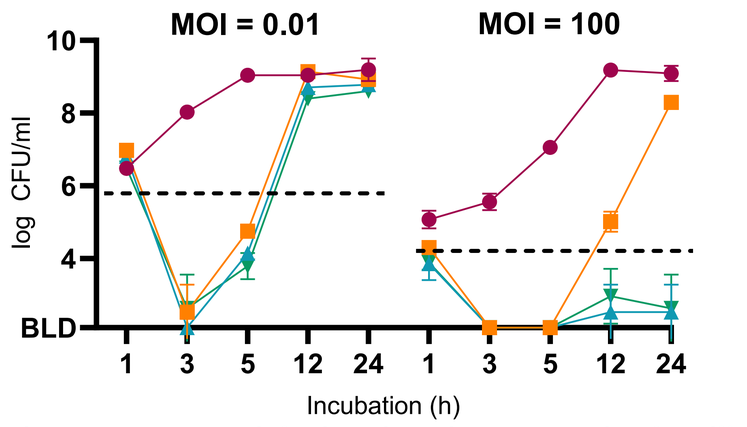

Figure 2: Bacterial titration in pasteurized milk. Samples were inoculated at MOIs of 0,01 and 100 and incubated during 1, 3, 5, 12 and 24 h at 37°C. Red = control (bacteria in milk), Orange = ULIVPec1, Blue = ULIVpec2, Green = ULIVPec1+2. BLD = below the limit of detection

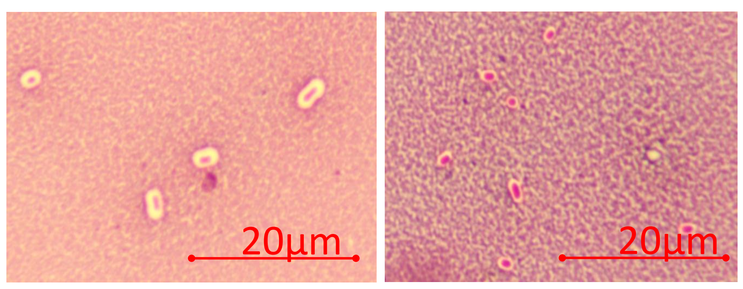

Figure 4: Maneval staining of *E. coli* strain 823 (left) and strain m823_G (right).

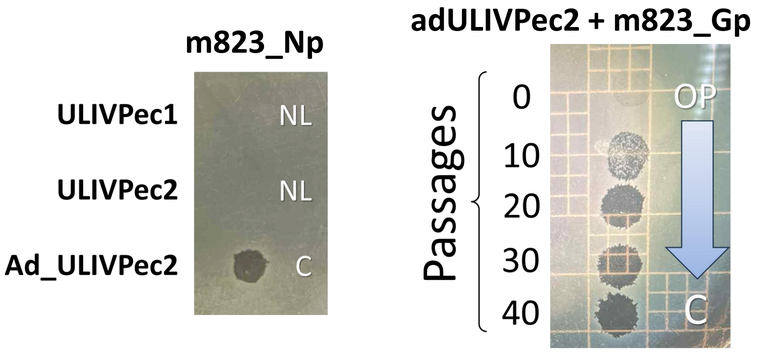

Figure 5: Host range expansion of ad_ULIVPec2.
Left: Lysis of the adapted phage against m823_Np
Right: Host range improvement against m823_Gp occured with the number of passages

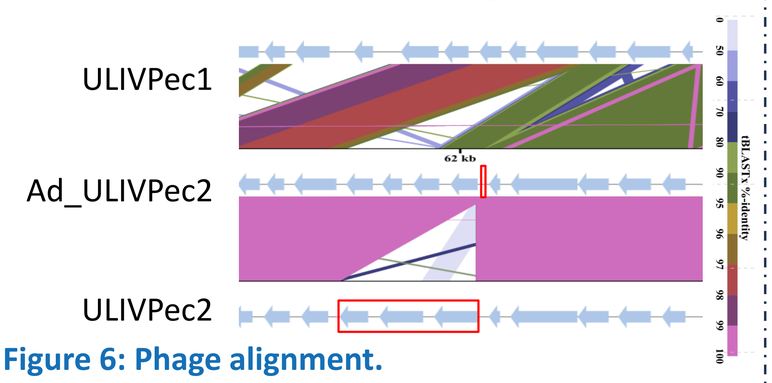

Figure 6: Phage alignment.

Bacterial staining

MM: The Maneval staining was used to highlight the bacterial capsule (white halo).

Res: All resistant strains presented à thinner capsule than the wt strain (**Figure 4**). Some mixed population were observed.

Phage adaptation

MM: Phage adaptation was conducted using the Appelman protocol on a cocktail of ULIVPec1 and 2 (1:1 ratio). The cocktail was co-cultured during 40 passages with 8 different strains, with 4/8 strains being resistant to the phages. The adapted phage was purified and sequenced with Illumina.

Res: The adapted phage ad_ULIVPec2 showed an improved host range against 3 strains (**Table I and Figure 5**), which included a strain that was not used in the Appelman protocol. Genome sequencing showed that ad_ULIVPec2 is derived from ULIVPec2. Genome alignment highlighted a deletion of 3 juxtaposed genes in the adapted phage (**Figure 6**).

Phage adsorption

MM:

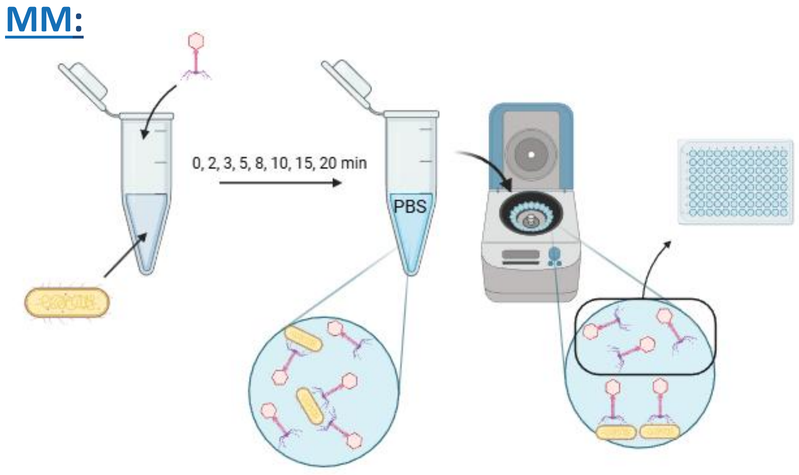

Figure 7: Determination of the phage adsorption.

The adsorption time was determined when 90 % of the initial phages were attached to the bacteria.

Res: The wt strain was adsorbed in less than 2 minutes on all phages. No mutant was adsorbed within 20 min.

Table I: Spot test of bacteriophages on different bacterial strains.

Phages	WT strain	Isolated mutant strains							Passed strains				Other
	823	m823_B	m823_C	m823_D	m823_G	m823_J	m823_K	m823_N	m823_Dp	m823_Gp	m823_Jp	m823_Np	847
ULIVPec1	C	NL	C	NL	NL	NL	NL	NL	C	OP	OP	NL	C
ULIVpec2	C	NL	C	NL	NL	NL	NL	NL	C	OP	OP	NL	C
ad_ULIVPec2	C	NL	C	NL	NL	NL	NL	NL	C	**C**	**C**	**C**	C
Appelman	X		X	X	X				X	X		X	X

Host range comparison of phage ULIVPec1 and 2 with the adapted phage ad_ULIVPec2 on the wt (wild-type) and mutant strains. The strains used for the phage adaptation (Appelman) are identified with a cross. C = confluent lysis, OP = opaque lysis, NL = no lysis.

Conclusion: Bacterial resistance is a rapid process that impairs phage efficacy. The underlying mechanisms may involve modifications of bacterial receptors, as suggested by the Maneval staining and the adsorption assay, and will be clarified by genome sequencing. Regarding phage ad_ULIVPec2, it may have adapted by evading recognition by the bacterial defense system through the deletion of 3 genes. Further *in vivo* experiments in *Galleria mellonella* model will compare the virulence of mutant strains and assess the efficacy of both wt and adapted phages on the larvae survival.

Acknowledgements: This program benefited from a financial support of Wallonia in the frame of a BioWin's Health Cluster and Wagralim's Agri-food Innovation Cluster program (Vetphage)

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