Extracellular vesicle formation in *Lactococcus lactis* is stimulated by prophage-encoded holin-lysin system

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Background

As a number of pathogens, probiotics and fermentation starters are Gram-positive bacteria, Gram-positive bacterial extracellular membrane vesicles (EVs) have been drawing attention in recent years for their possible roles in human health and disease. However, mechanistic insights are still lacking on how EVs are released through the cell walls in Gram-positive bacteria, as the peptidoglycan layer had been historically presumed to be a strong physical barrier preventing such event. In this study, we characterized underlying mechanisms of EV production and provide evidence for a role of prophage activation in EV release using the Gram-positive *Lactococcus lactis* as a model.

Strains

1. Artisanal cheese starter culture isolate, *L. lactis* **FM-YL11** (lysogen, containing a prophage in the genome)
2. Prophage-cured derivative **FM-YL12**
3. Prophage-encoded holin-lysin knockout mutant **FM-YL11ΔHLH**

Treatment

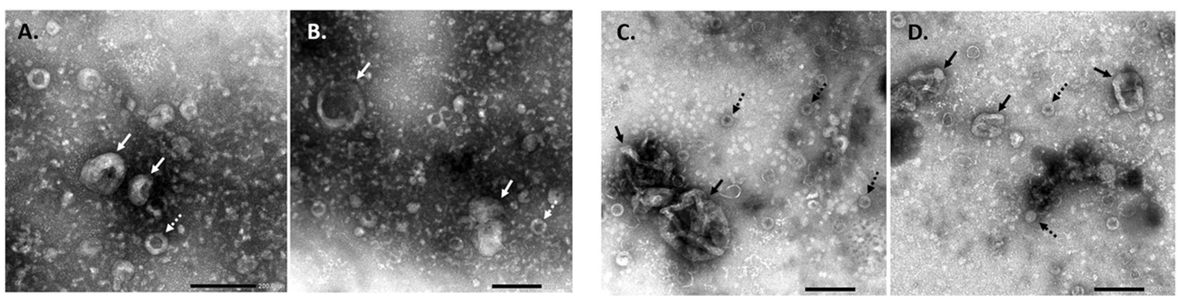
L. lactis strains cultivated in M17 (0.5% (wt/vol) lactose), exposed to:

1. **Prophage-inducing condition:** addition of 1 µg/mL mitomycin C
2. **Non-inducing condition:** absence of mitomycin C

Results

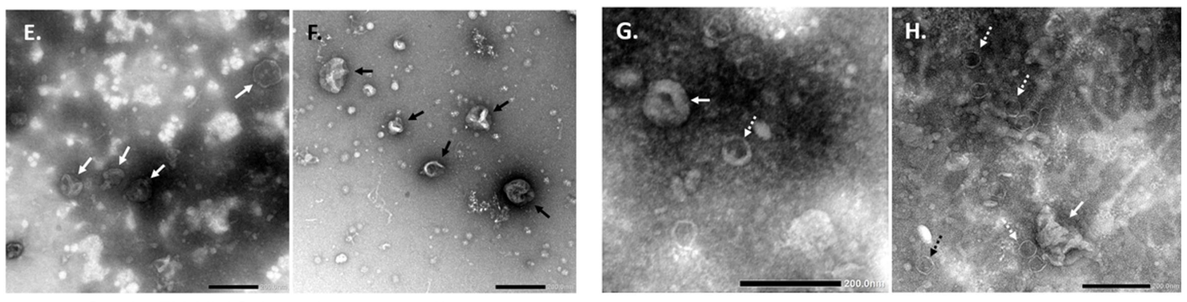
We applied standard EV isolation procedures (microfiltration + ultracentrifugation) to the culture supernatants of all strains under the prophage-inducing and non-inducing conditions. Using electron microscopy (TEM), EV-like structures were observed in all samples, while phage head-like structures were only observed samples from the lysogenic strains.

EV-like structures | Phage head-like structures | All scale bars = 200 nm



Strain FM-YL11, prophage-inducing condition

Strain FM-YL11, non-inducing condition

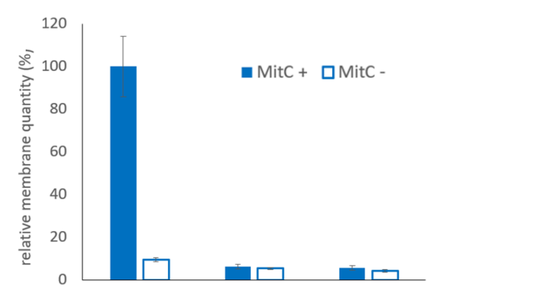


Strain FM-YL12 (prophage-cured), prophage-inducing condition

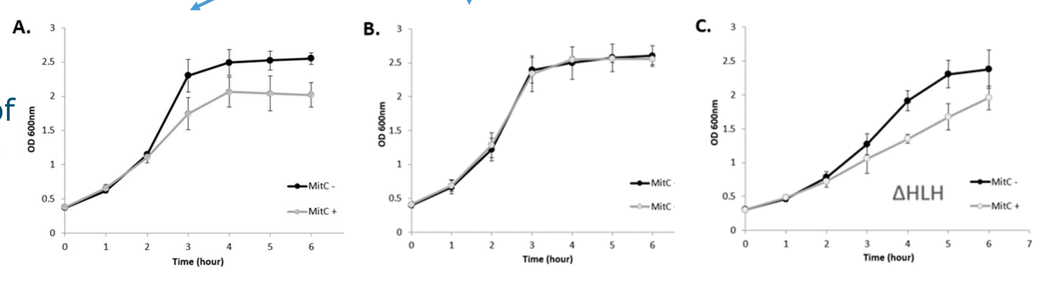
Strain FM-YL11ΔHLH (holin-lysin KO), Prophage-inducing condition

EV quantification with membrane-specific dye FM4-64 reveals 10-fold higher EV quantity in FM-YL11 culture under the prophage-inducing condition compared to non-inducing condition, which was not observed in the two other strains. Moreover, EV release in all cases does not induce massive cell lysis, as reflected by the OD growth curves.

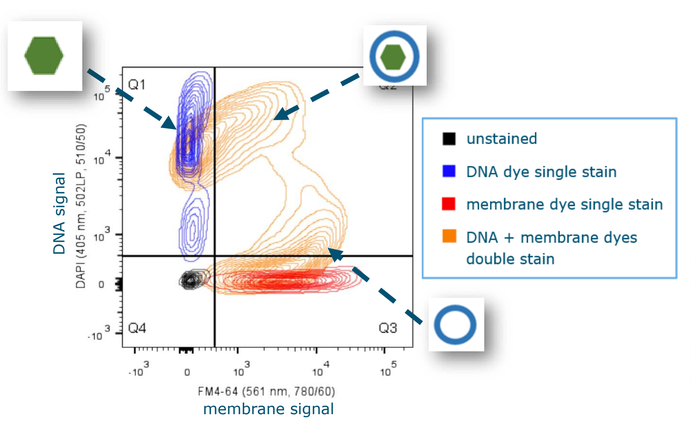
EV quantification

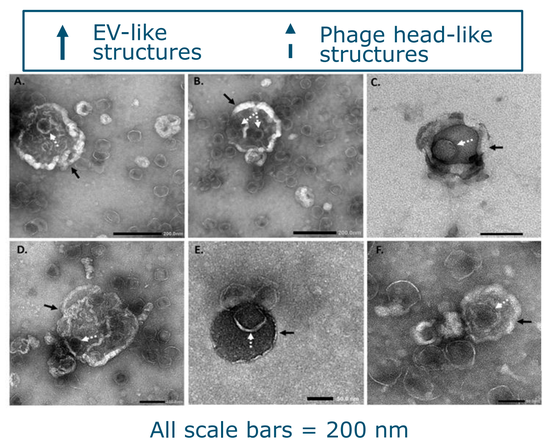


Growth of bacteria



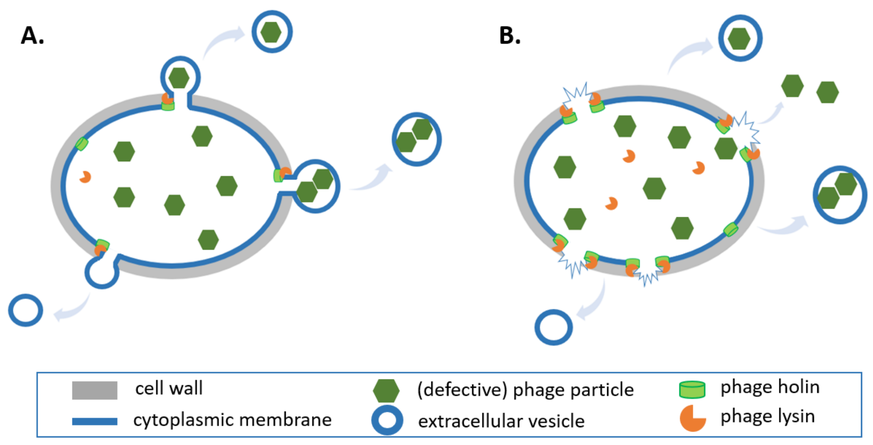
Flow cytometry analysis on the holin-lysin induced EVs from FM-YL11 with DNA or/and membrane specific dyes suggest presence of a population of EVs that contains phage particles as cargo. Structures that are likely EV-enclosed phage heads could indeed be observed by TEM.





All scale bars = 200 nm

We propose a model for holin-lysin induced EV release in L. lactis via two scenarios: **A)** EVs generated by "budding" without immediately lysing the cells of the major population, and **B)** EV formation upon lysis of a minor population of cells. In both scenarios EVs may encapsulate phage particles as cargo.



Conclusions

- Gram-positive *L. lactis* produces EVs.
- Prophage-encoded holin-lysin system mediates massive release of EVs.
- Phage particles are likely cargos of EVs.

Outlook

- Studies on Gram-positive EVs contribute to understanding and applications in human health and disease.

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