

TITLE: DISTINCT PATHWAYS INVOLVED IN OUTER MEMBRANE VESICLES BIOGENESIS IN *Chromobacterium violaceum*

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ABSTRACT:

Outer membrane vesicles (OMVs) are nanoparticles released by Gram-negative bacteria, which play pivotal roles in bacterial physiology, host-pathogen interactions, and adaptation to diverse environments. Although the knowledge regarding OMVs has largely increased, many questions remain elusive. In this work, we characterized pathways involved in OMVs biogenesis in the Gram-negative opportunistic pathogen *Chromobacterium violaceum*. Using ultracentrifugation, dynamic light scattering, protein content, dot blot, and transmission electron microscopy, we revealed that *C. violaceum* releases OMVs that have a diameter in a range of 50-300 nm. We showed by HPLC and mass spectrometry analysis that OMVs from *C. violaceum* contain violacein, a well-known metabolite with antimicrobial properties. Purified OMVs from the wild-type strain inhibited Gram-positive bacteria, while OMVs from the mutant Δ *vioABCDE*, which lacks violacein, were unable to kill these target bacteria. Furthermore, Δ *vioABCDE* produced 2-fold fewer OMVs than the wild-type strain, indicating that violacein induces OMVs biogenesis. Thus, OMVs production induced by violacein allows *C. violaceum* to deliver a hydrophobic compound against other bacteria. Additionally, we quantified OMVs production in *C. violaceum* Δ *vacJ* and Δ *yrbE* mutants. The VacJ/Yrb transport system modulates OMVs production by controlling the outer membrane asymmetry in Gram-negative bacteria. As expected, *C. violaceum* Δ *vacJ* and Δ *yrbE* were hyper-vesiculating strains. As OMVs secrete LPS, we hypothesized that Δ *vacJ* and Δ *yrbE* could trigger host immunity faster than wild-type, leading to a faster infection resolution. In agreement, Δ *vacJ* and Δ *yrbE* had its virulence attenuated in mice. Moreover, Δ *vacJ* and Δ *yrbE* displayed regular cell shape and growth, as verified by transmission electron microscopy and growth curves in LB, minimal medium and after treatment with Polymyxin B. Lastly, our work also revealed that a mutant strain Δ *dsbA*, previously described as a hypo-vesiculating strain in *E. coli*, curiously displayed a hyper-vesiculating phenotype in *C. violaceum*, suggesting that the disulfide bond formation protein DsbA could have a distinct role in bacterial vesiculation. The mutant strain Δ *dsbA* showed an attenuated virulence phenotype in mice. In conclusion, we described three distinct mechanisms for OMVs biogenesis in *C. violaceum* that could trigger OMVs release in different contexts, contributing to interbacterial competition and pathogenesis.

Keywords: Outer membrane vesicles, *Chromobacterium violaceum*, violacein, LPS, host-pathogen interactions.

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