Comparative Structural-Antibiotic binding Analysis of Lol Proteins in Gram-Negative Pathogens and Discovery of a LolB-Like Protein in *P. gingivalis* Using Alphafold

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Abstract: The bacterial cell envelope plays a critical role in maintaining viability, growth, and pathogenicity. Gram-negative bacteria, unlike gram-positive bacteria, possess double cell membranes, with the outer membrane often associated with pathogenicity and antibiotic resistance. Lipoproteins, crucial structural and functional components of the cell envelope, perform various physiological functions and have virulence-associated roles such as antigenicity and colonization. **L**ocalization **o**f **l**ipoproteins is essential for outer membrane biogenesis, a process mediated by the Lol pathway, comprising an integral membrane complex LolCDE (ABC transporter), a periplasmic chaperone LolA, and an outer membrane-anchored receptor LolB. While LolB is responsible for transferring lipoproteins from LolA to the inner leaflet of the outer membrane, its absence in certain bacterial species, including *Porphyromonas gingivalis*, raises questions about alternate mechanisms for lipoprotein transfer.

In this study, we solved the crystal structures of LolA from *Vibrio cholera, Porphyromonas gingivalis*, and *Helicobacter pylori*, along with LolB from *Vibrio cholera*, broadening the repertoire of LolA/B structures across gram-negative bacteria. Our structural analysis revealed distinct features and binding specificities among LolA and LolB homologues. Interestingly, LolB from *Vibrio cholera* showed selective binding only with LolA from the same species, not with LolA from *P.gingivalis*. Furthermore, we performed isothermal titration calorimetry (ITC) analysis to assess the binding affinities of Lol proteins with antibiotics Polymyxin B, Polymyxin E, and Polymyxin Nonapeptide. Our results showed that LolA from *P.gingivalis* and *V.cholera* exhibited the highest affinity for Polymyxin B, followed by Polymyxin E and with weak binding to polymyxin nonapeptide. Conversely, LolA from *H.pylori*, despite having a larger accessible cavity, showed no binding to any of the tested antibiotics. The differential binding of polymyxin derivatives lays foundation to develop pharmacophore model for identifying polymyxin-like or novel compounds. Additionally, the inability of LolA from *H.pylori* to bind to any of the tested antibiotics underscore the necessity of parameterizing protein-centered scoring functions to minimize false positive hits in virtual screening.

To address the absence of LolB in certain bacterial species, including *P.gingivalis*, we analyzed the *P.gingivalis* proteome using Alphafold2 and identified a protein predicted to be structurally similar to LolB. We purified this protein, solved its crystal structure, and demonstrated its interaction with LolA from *P.gingivalis* via ITC and BLI, confirming it as the cognate binding partner of LolA *P.gingivalis*.

Our structure determination and affinity measurements of Lol proteins with available antibiotics provide an experimental foundation for steering drug development in a targeted direction. Furthermore, our study highlights the importance of combining proteomic analysis with Alphafold2 to identify functionally and structurally homologous proteins that may have been missed using conventional sequence homology searches.