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Structural Analysis of *Helicobacter pylori* VacA's Channel in Membrane

Sarah M. Connolly[1, 2], Amanda L. Erwin[1], Megan Sabb[1], Georgia Caso[3], Timothy L. Cover[3, 4, 5], and Melanie D. Ohi[1, 2, 6*]

[1.] Life Sciences Institute, University of Michigan, Ann Arbor, MI, USA.
[2.] Cellular and Molecular Biology Program, University of Michigan, Ann Arbor, MI, USA.
[3.] Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA.
[4.] Department of Pathology, Microbiology, and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA.
[5.] Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN, USA.
[6.] Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, USA.
* Corresponding author: mohi@umich.edu

Helicobacter pylori is a Gram-negative bacterium that infects the human stomach and causes gastric inflammation [1]. *H. pylori* colonizes the stomachs of over half of the world's population and, in addition to chronic gastritis, can result in the development of peptic ulcer disease (PUD) and gastric cancer [1, 2]. Gastric cancer is the fourth leading cause of cancer-related deaths worldwide, and *H. pylori* is classified as a Type I carcinogen by the World Health Organization [3, 4].

Vacuolating cytotoxin A (VacA) is a key virulence factor expressed by *H. pylori* that increases the risk of developing peptic ulcer disease (PUD) and gastric cancer [5]. Following secretion by *H. pylori*, VacA binds to the membranes of gastric epithelial cells, oligomerizes, and forms anion-selective channels on various cell membranes [6]. Upon binding to membrane and forming its channel, VacA is proposed to induce a number of intercellular effects including vacuolation, membrane permeabilization, autophagy, and disruption of epithelial tight junctions [6, 7]. These intercellular effects depend on the formation of VacA's active transmembrane channel [6].

VacA is secreted by *H. pylori* as an 88kDa monomer (p88) consisting of an N-terminal p33 region and a C-terminal p55 region [6]. The p33 region contains a hydrophobic stretch that is required for channel formation [8]. This hydrophobic region contains three tandem GXXXG repeats that are predicted to form an α-helical secondary structure [9]. Deletion of this region results in mutant VacA that is unable to vacuolate mammalian cells and exhibits ineffective channel activity in lipid bilayers [8]. Despite the determination of VacA's soluble structure using single-particle cryo-electron microscopy (cryo-EM), the structure of VacA's channel region remains unknown due to a lack of defined density for the hydrophobic channel region in the soluble VacA structure [10, 11]. Given the importance of VacA's transmembrane channel activity for its cellular effects, determining the channel structure and mechanism of channel insertion is critical to further functional understanding of VacA's role in *H. pylori* infection.

To address the structural basis of VacA's channel activity in pathogenesis, we analyzed VacA in the membrane context of liposomes using single-particle cryo-EM and subtomogram averaging. 2D classification of VacA bound to small unilamellar vesicles (SUVs) revealed that VacA oligomers interact with the SUV membrane but fail to insert fully into the lipid bilayer. This analysis suggests that VacA exhibits a hemipore state where its hydrophobic regions are partially inserted into membrane but the full transmembrane channel is not formed. Using cryo-electron tomography (cryo-ET), we investigated the formation of VacA channels in liposomes. Using subtomogram averaging, we

determined a mid-resolution structure of VacA's channel-inserted state. These data together suggest that, upon binding membrane, VacA undergoes an initial transition to a partially inserted channel state before full insertion of its channel.

This study offers significant insights into the structural basis of VacA's transmembrane channel and the mechanism of channel insertion into membrane. Given the importance of VacA's channel in intercellular effects, this study provides a structural understanding of channel insertion that will be used to inform studies on VacA's function in the context of pathogenesis [6, 12].

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