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Microcolony formation as a stage in adherence of nontypeable *Haemophilus influenzae* to human lung cells

Nontypeable (nonencapsulated) strains of *Haemophilus influenzae* (NTHi) are responsible for respiratory diseases ranging from childhood otitis media to pneumonia. Long-term persistence of NTHi in respiratory tissue is associated with biofilm formation and antibiotic resistance in chronic lung disease and recurrent otitis media. We are interested in early stages of bacterial association with lung tissue. Initial adherence (within 30min) to tissue culture cells is known to be mediated by one of several high molecular weight adhesins; however, over a period of 4h binding becomes more efficient. The phase-variable autotransporter Lav, an outer membrane protein of many NTHi, improves binding in this second stage although it is not required for initial adherence. When diluted NTHi are allowed to adhere to H292 lung carcinoma cells for 4h, microcolonies of about 50 bacteria are visible in the SEM or by fluorescence microscopy with GFP-labeled bacteria. In the confocal microscope, points of microcolony formation coincide with regions of polymerized F-actin stained with rhodamine-labelled phalloidin. These polymerized actin structures are reminiscent of those induced in localized adherence to gut epithelium by diarrheogenic E. coli. Since the number of bacteria in a microcolony exceeds the number expected for clonal growth from a single bacterium, we hypothesize that NTHi are being recruited to localized points of adherence. To test this hypothesis, we have constructed isogenic RFP-labeled and GFP-labeled NTHi to use in mixing experiments with dilute bacteria. If microcolonies are polyclonal, we expect to find mostly mixed red and green colonies in association with polymerized actin structures; if they have grown from a single cell most colonies will be either red or green. We have also constructed phase-locked ON Lav mutants for comparison of longer-term adherence with Lav knockouts.