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Using Negative Staining TEM to Study Structure/Function Relationships of Cystic Fibrosis Host-Adapted Opportunistic Pathogen *Pseudomonas aeruginosa*

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Development of improved antimicrobial therapies for patients with cystic fibrosis (CF) who endure frequent, persistent and potentially life-threatening chronic infections with opportunistic pathogens, is sought [1-3]. One approach, in our laboratories [4], is trained on interfering with host-pathogen carbohydrate-binding interactions that first involves profiling the carbohydrate binding of clinical isolates of *Pseudomonas aeruginosa* (PA), a key player in CF infections [1,2,5]. This adaptive organism is known to modulate its virulence factor production during colonization and under differing growth conditions [1,2]. Wild type PA typically present with motile phenotypes on plate culture, while CF patient isolates are often non-motile or mucoid/non-motile expressing a dense protective extracellular matrix. To address what features a variety of PA might present to our binding studies, and to permit clearer assessment how these attributes correlate functionally with other laboratory binding observations, we required an imaging technique which could be applied to our specimens as they go into the binding assays. Results show that a negative stain approach using an organotungsten stain, with minimal manipulations of cultures prior to transmission electron microscopy (TEM) analysis, provides for quality images of general population characteristics and permits survey of presence or absence structural features associated with adherence, such as flagella and pili [1,5].

A diverse collection of PA was used, including clinical isolates from CF and non-CF patients (NCF), and laboratory strains from the American Type Culture Collection (ATCC). PA were typically cultured on solid agar (blood agar, BA) or in liquid culture (minimal media with glucose, MM). Agar grown bacteria were gently suspended in sterile filtered distilled water, maintaining consistent and limited time in water to avoid swelling artifacts. Liquid cultured PA were harvested by gentle centrifugation (*i.e.* 2 min, 2000 x g) then suspended in water. Just prior to use, bacterial solutions were gently mixed to uniform distribution. Glow-discharged formvar/carbon-coated Cu grids were floated atop 25 µl drops of bacterial suspensions for 3-5 minutes. After excess solution was gently wicked off, grids were placed on 10 µl drops NANO-W solution, (methylamine tungstate, pH 6.8, Nanoprobes), stained for 3-5 minutes and, following the careful removal of excess solution, were air dried. At this point, specimens were analyzed directly or stored in grid boxes until analysis. Images were acquired on a standard biological TEM (JEOL JEM-1400 TEM, at 120kV), with DigitalMicroscope software (Gitan).

This protocol permitted comparisons of strain characteristics at the TEM level from a variety of patient sources, plate culture phenotypes, and culture conditions. Similar to phosphotungstic acid staining [5], flagella, and pili were readily imaged from organotungsten stained wet-mounts (Fig. 1). This strategy yielded high contrast and fine detail with very little non-specimen background. For pili, which are not uniformly distributed within pili "+" culture populations, higher magnifications confirmed structures. Attention to the appropriate and consistent amount of bacteria in suspension, and time in water and stain, all contributed to successful imaging. As such, this technique proved to be a convenient approach with a brief preparation time from 'culture-to-image'. The panel of PA evaluated here, which are phenotypically diverse in plate culture, also showed heterogeneity at the EM level (Figs. 2,3). Flagella were readily observed for most motile specimens, such as wild type ATCC-BAA-47 Fig. 2A, but not all.

Pili alone appeared sufficient for motility for one NCF-sputum isolate (Fig. 2B). To our surprise, many of the non-motile (nm) and mucoid/nm CF airway PA isolates possessed flagella (*i.e.* Fig. 2C, nm; Fig. 2D, muc/nm). Analysis of liquid cultures generally revealed structural features consistent with solid agar cultures of the same organisms and showed marked diversity of growth styles (Fig. 3). Use of TEM in this way to assess *in vitro* clinical isolate characteristics, the degree of preservation, during processing, of native bacterial structures for binding assays, and the effects of culture conditions, provided the means to correlate structural features with functional and behavioral characteristics seen in other types of laboratory investigations.

In conclusion, negative staining as described with TEM imaging, provides a convenient, and invaluable tool to 'visually' assess and compare structural and population characteristics of phenotypically heterogenous isolates of *Pseudomonas aeruginosa* that may confirm, augment, or even contrast, gene or protein expression information about host-adapted bacteria.

References:

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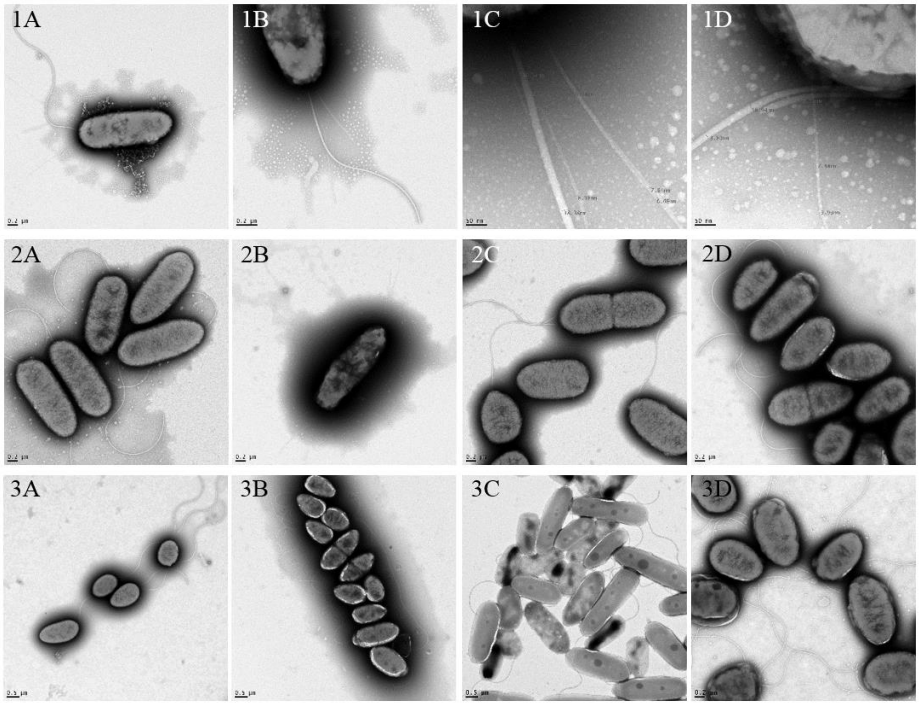

Figure 1. NANO-W stained *P. aeruginosa* (PA). Blood agar grown: (A-C) CF-T 3435, motile (mot), flagella (F) +, pili (P) +; (D) CF-S 8314-1, non-mot (nm), F+, P+. Scale bars: (A,B): 0.2 μm; (C, D): 50 nm.

Figure 2. Plate grown motile and non-motile PA with varying presentations of flagella and pili. (A) ATCC BAA-47, mot, F+; (B) NCF-S 3391, mot, F-, P+; (C) CF-S 8314-1, nm, F+ (P+, Fig. 1D); (D) CF-S 8314-2, muc/nm, F+ (P+). Scale bars: 0.2 μm.

Figure 3. Minimal media (MM) liquid culture grown CF sputum PA isolates with varied growth habits. (A) CF-S 8314-1 (nm), dispersed; (B) CF-S- 8314-2 (muc/nm), multiply as group in matrix; (C) CF-S 3318 (mot), poor MM growth; (D) CF-S 8981-2 (muc), present as groups, demonstrably F+, P+. Scale bars: (A,B,C) 0.5 μm; (D) 0.2 μm.

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