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HETEROGENEITY OF GENE EXPRESSION AND ACTIVITY IN BACTERIAL BIOFILMS

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Introduction

Putative differences in physiology between planktonic microbial cells and their attached counterparts (cells grown in biofilms) form the basis for several important hypotheses regarding the behavior of biofilm cells. For example, it is generally accepted that cells in biofilms are at least ten-fold more resistant to antimicrobials than are cells from liquid culture. One theory explaining the higher resistance is that, because the mode of action of most antimicrobials in fact requires growth, a lower level of metabolism (growth) occurs in biofilm-grown cells than in planktonic cells and that higher doses of antimicrobial are required to affect these slower metabolizing cells (1). Despite the critical importance of a general knowledge of how cells in biofilms metabolize, the regulation of gene expression and metabolism in microorganisms associated in biofilms (i.e., attached to substrata) is largely undescribed; this is partially a result of only recent development of methodology to study single-cell or microcolony-level gene expression within living biofilms. The potential use of anchored cells as biosensors to monitor pollutant levels (2) and gauge cleanup efforts in real time also spurs interest in time-resolved, non-destructive quantitation of gene expression by attached cells. In this paper, we employ a biofilm-grown bacterium (*Pseudomonas putida*), genetically engineered to contain chromosomally integrated coupled toluene-degradation (*tod*) and luciferase (*lux*) genes, to monitor the presence of toluene in a flow-through system. Correlative microscopy (use of more than one microscopic technique on the same specimen) combining photon-counting microscopy to monitor light production (equals toluene degradation) with laser confocal microscopy to assess microbial activity demonstrated that response to toluene removal has a three-hour lag phase and that individual microcolonies within the clonal biofilm have varying levels of gene expression and of membrane potential.

Materials and Methods

Bacterium *Pseudomonas putida* strain TVA8B was transformed using a miniTn5 vector containing the *tod/luxCDABE* gene fusion resulting in cells capable of growth with toluene as the sole carbon source (3). Cells were grown overnight in LB broth on a shaker at 23° C; 2 h prior to experiments these cells were diluted 100-fold into minimal medium (in g/L: KH_2PO_4, 0.68; K_2HPO_4, 1.73; $MgSO_4 \cdot 7H_2O$; NH_4NO_3, 1.0; plus trace elements) plus 10 µg/mL glucose and allowed to regrow on the shaker for 2 h.

Flowcell Experiments Flowcells (glass perfusion chambers for microscopy of biofilms) (4) were filled by injecting 200 µL of a ten-fold dilution (in minimal medium) of the glucose-metabolizing cell suspension. After a 20 min attachment period, a low-volume precision peristaltic pump (#205, Watson Marlow, Wilmington MA) was used to continuously deliver 185 µL/min of minimal medium plus 100 µg/L toluene. When desired, minimal medium plus 10 µg/L glucose was pumped through the flowcell to remove toluene; after switchover, residence time of toluene-containing medium in the 200 µL volume of the flowcell was extremely short relative to the period of light measurement (see below).

Photon-counting Microscopy of *tod* Gene Expression A photon-counting camera (VIM3 camera and Argus 50 control software, Hamamatsu Photonics, Bridgewater NJ) was mounted on a Leica DMR microscope and the biofilm was observed with a 100x 1.3 N.A. oil-immersion lens (5). Light output from microcolonies was measured over a period of 5 min each hr. Background counts were determined in a region that contained no cells. The total area of cells at the substratum of each microcolony was estimated by digital image analysis (Ultimage, Graftek Imaging, Mystic CT) of transmitted light images collected immediately prior to the photon-counting images. Light production was normalized by subtraction of background counts from counts acquired from an individual microcolony, then division of that number by the area of microcolony. Light output by microcolonies differing in size (x-y dimensions) could be directly compared; the colonies varied little in height (see below). For the toluene-removal experiment, results are expressed without correction for colony area.

Laser Confocal Microscopy of Overall Cellular Activity Tetramethylrhodamine methyl ester (TMRME; Molecular Probes, Eugene OR), a stain used to quantify mitochondrial membrane potential (6), was injected into the flowcell at a concentration of 1 mg/mL (in minimal medium plus glucose) at the end of one experiment. After a 5 min reaction period, unbound stain was washed out and laser confocal images (568 excitation, > 580 emission; see figure for details) were acquired by replacing the photon-counting camera with the Leica TCS-NT confocal scanhead. We interpret cells with high fluorescence (much dye binding) as having high membrane potential (metabolic activity) *vs.* those cells with low fluorescence.

Results and Discussion

Figure 1 shows that light is measurable from colony C within 1 hr after induction. Light production from colony B does become significant until 2.5 hr, and colony A never makes significant light over the period of the experiment.

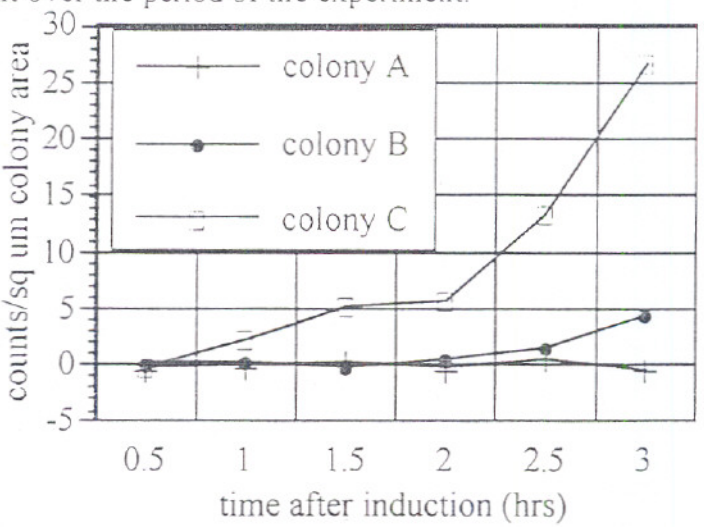

Figure 1. Biomass-normalized light production from three different microcolonies after induction by toluene.

The transmitted light images in Figure 2 demonstrate that colony A does not grow as rapidly as do the other colonies, and the photon-counting images (Figure 2) show that colony C has two distinct areas of light production.

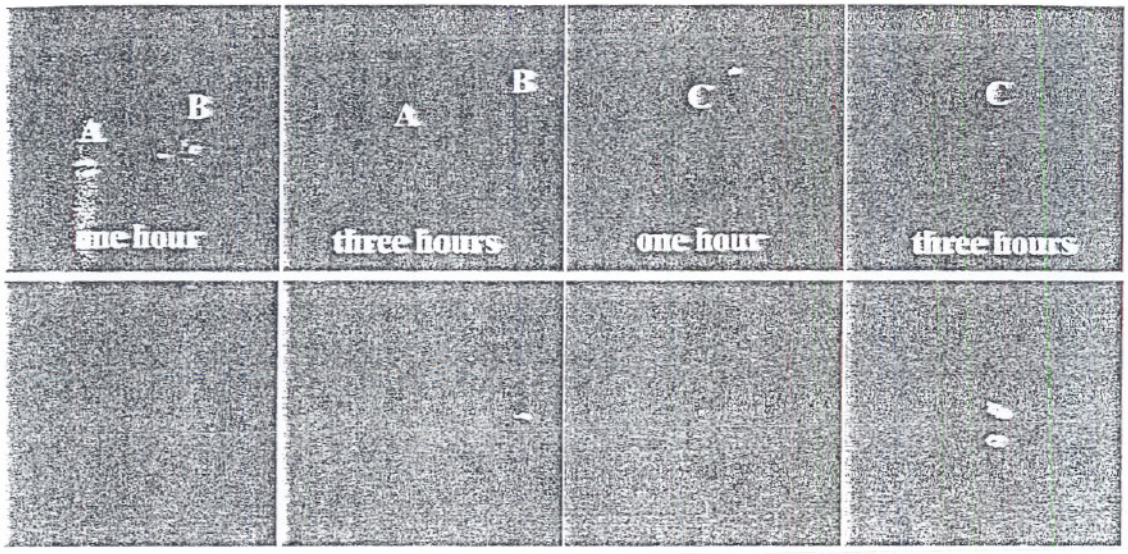

Figure 2. Transmitted light images (top row) and photon-counting images (bottom row) of colonies from Figure 1. Colony A does not grow and colony C has two regions of light production.

The low-magnification TMRME fluorescence image of all the colonies within the same field of view (Figure 3a) clearly shows that colonies B and C are brighter than colony A; however, two relatively bright spots can be seen in colony A. High-magnification images of the individual colonies (Figure 3b,c,d) show that colony A has two bright cells whereas the other colonies have many bright cells. These data demonstrate that colony A produces little light because it contains few (perhaps only two) active cells; most of the cells in this colony do not grow nor do they metabolize toluene over the course of this experiment. Colonies B and C each contain many active cells; light is produced and colony size increases. Colony C contains distinct locations from which light emanates; these locations do not contain more cells (as determined by examination of individual confocal sections from the colony, data not shown), nor is TMRME fluorescence greater than that of cells from surrounding areas. It appears that low membrane potential can explain low light production, however it cannot be used to distinguish between cells that are producing light. Furthermore, light production within this presumably clonal bacterial biofilm is heterogeneous and controlled at the level of the single cell; metabolic differences exist between microcolonies and between individual cells within a single microcolony in biofilm-grown bacteria, and individual bacteria within a biofilm may not respond identically to a given effector. These ideas lend support to the hypothesis that biofilm-grown cells have distinct metabolic differences from their planktonic counterparts, and they also lead to the corollary that these differences can be seen even from cell to cell.

Down-regulation of *tod* expression was tested by removing toluene from light-producing biofilms. Figure 4 shows that the light production of two different colonies responded identically to removal of toluene, that there was a lag period of 3 hours prior to decrease in light production, and that the rate of decrease during down-regulation was the same as the rate of increase during induction. These data suggest that the turnover time of luciferase in the cell is about three hours, or that the response time of the *tod* gene to down-regulation has a significant lag time in biofilm-grown cells. We hypothesize that the latter is true; that the *tod* (and *lux*) mRNA transcripts persist in the cell over this lag period.

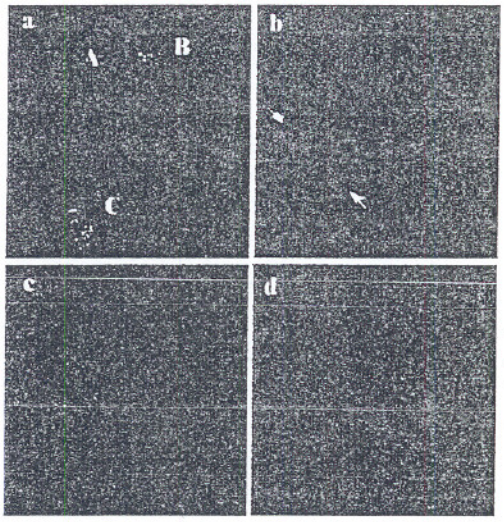

Figure 3. Laser confocal images of TMRME fluorescence of colonies from Figures 1 and 2.
a) All three colonies. Image is 100 µm x 100 µm. Seven sections through 2.4 µm depth.
b) Colony A. Image is 25 µm x 25 µm. Three sections through 1.4 µm depth.
c) Colony B. Image is 25 µm x 25 µm. Nine sections through 3.2 µm depth.
d) Colony C. Image is 25 µm x 25 µm. Five sections through 2.8 µm depth.

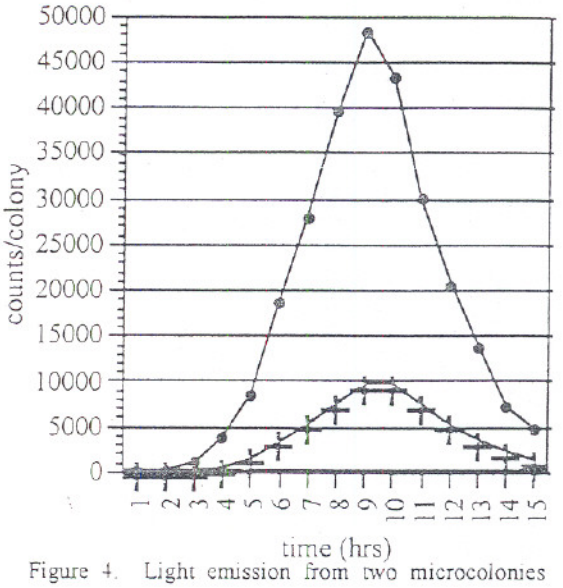

Figure 4. Light emission from two microcolonies during down-regulation of tod/lux genes. Toluene was present from time zero and was removed at six hours.

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