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A combination of assays reveals biomass differences in biofilms formed by *Escherichia coli* mutants

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Abstract

Aims—The aim of this study was to develop an assay system that can quantify the amount of biomass in biofilms formed by different isogenic mutants of an *Escherichia coli* K-12 strain.

Methods and Results—The reported assay, which is based on the BacTiter-Glo™ assay from Promega, uses bioluminescence to detect the intracellular concentration of ATP, which correlates with viable bacterial cell numbers. The quantitative data obtained with this ATP assay were compared to those obtained with the conventional crystal violet assay. As a qualitative control, scanning electron microscopy was performed.

Conclusions—The ATP assay, the crystal violet assay, and scanning electron microscopy yielded similar results for six of the eight strains tested. For the remaining two strains, the images from the scanning electron microscopy confirmed the results from the ATP assay.

Significance and Impact of Study—The ATP assay, in combination with other quantitative and qualitative assays, will allow us to perform genetic studies on the regulatory network that underlies the early steps in *E. coli* biofilm formation.

Keywords

biofilm; live biomass; quantitative assay; ATP; crystal violet; flagella; type I fimbriae; extracellular matrix

Introduction

Biofilms, sessile communities of bacteria encased in a matrix, exert a profound impact in many natural (Ferris et al. 1989; Nyholm et al. 2002), clinical (Nicolle 2005; Rice 2006), and industrial settings (Brink et al. 1994; McLean et al. 2001; Wood et al. 2006). Biofilms develop on surfaces in a series of ordered steps mediated by surface organelles (Sauer et al. 2002). In *Escherichia coli*, the first step, reversible attachment, is mediated by flagella. The next step is irreversible attachment. It requires type 1 fimbriae and the loss of flagella. The third step, formation of the mature biofilm, requires production of an extracellular polysaccharide matrix, such as colanic acid (for a review, see (Van Houdt and Michiels 2005). The coordinated expression of these surface organelles requires the involvement of

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several transcriptional regulators including FlhD/FlhC, OmpR, and RcsB (reviewed by (Prüβ et al. 2006).

Successful dissection of this complex biofilm-associated regulatory network requires a quantitative assay that can complement microscopic visualization techniques. For this purpose, many assays have been developed (for a comparison, see (Peeters et al. 2008). For example, crystal violet (CV) and Syto9 detect extracellular matrix polymers, staining live and dead cells alike, while 2,3-bis (methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) and resazurin detect only live bacteria. Because of its wide range of applicability, the CV assay has been developed for high-throughput studies (Stafslien et al. 2007; Stafslien et al. 2006). However, since it detects matrix, the CV assay may have limitations for studies, such as our own, that focus on the involvement of flagella and type I fimbriae in biofilm formation. The XTT assay also has limitations: it requires highly respirative bacteria (Roehm et al. 1991) and has been reported to experience intra- and interspecies variability (Kuhn et al. 2003).

ATP concentration is relatively constant across many different growth conditions (Schneider and Gourse 2004). For this reason, ATP-based assays are used routinely to detect bacteria. For example, ATP-based assays have been used to monitor the effect of antimicrobials on the live biomass in biofilms (Monzon et al. 2001; Romanova et al. 2007; Takahashi et al. 2007). More specifically, the ATP-based BacTiter-Glo™ assay has been used to measure attachment in *Pseudomonas aeruginosa* biofilms (Junker and Clardy 2007). In this study, we compare the quantitative data obtained with the BacTiter-Glo™ assay with data obtained with the conventional CV assay. We then evaluate the performance of both quantitative assays by comparison to scanning electron microscopy (SEM).

MATERIALS AND METHODS

Bacterial strains and growth conditions

All bacteria used in this study were isogenic derivatives of the *E. coli* strain AJW678, which is wild-type for acetate metabolism and the biosynthesis of flagella, type 1 fimbriae, and colanic acid (Kumari et al. 2000). The genes tested include *flhD* (flagellar master regulator, *flhD*::*kn*), *fimH* (fimbriae adhesin, *fimH*::*kn*), *ompR* (response regulator for osmoregulation, *ompR*::*Tn10*), *rcsB* (response regulator for capsule synthesis, *rcsB*::*kn*), *rcsC* (histidine kinase/phosphatase for capsule synthesis, *rcsC*::*Tn5*), *ackA* (acetate kinase, *ackA*::*TnphoA'-2*), and *ackA pta* (acetate kinase and phosphotransacetylase, Δ(*ackA pta hisJ hisP dhu*)). Strains were maintained at −80°C in 15% glycerol. Bacteria were plated onto Luria Bertani plates (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) and incubated overnight at 34°C. Liquid overnight cultures were prepared in tryptone broth (TB; 1% tryptone, 0.5% NaCl).

Formation of biofilms for the quantitative assays

Bacteria were diluted 1:100 from overnight cultures into 100 µl of TB dispensed into a 96 well polystyrene microtiter plate. The microtiter plate was incubated without shaking at 37°C for the indicated number of hours. The incubation period was 38 h for the single time point experiments and 2 to 48 h for the time course experiment. During this time, most bacterial strains formed a solid biofilm at the bottom of the well. As previously described (Wolfe et al. 2003), some strains also formed a pellicle that covered the entire surface of the culture. To harvest both biofilms, we removed the culture medium carefully with a pipette. Biofilms were then rinsed carefully with 100 µl of PBS. In a final step of the biofilm preparation, the excess PBS was removed and the biofilms were air dried for 10 to 15 min.

For single time point experiments, each strain was tested inthree independent experiments, each of which included twelve replicates. The mean and the standard deviation were determined across the three independent experiments. For the time course experiment, wild-type bacteria and the isogenic *ompR* mutant were tested in three independent experiments of eight replicates each. Again, the mean was presented across all three experiments. For the single time point experiment, the wild-type strain AJW678 was used as a reference.

ATP assay

100 μl of PBS was added to each well containing biofilm prepared as described above. The bioluminescence reaction was started by the addition of 100 μl of BacTiter-Glo™ reagent (Promega, Madison WI; prepared according to the manufacturer's instructions) to each well. Incubation time was 5 min at room temperature. Bioluminescence was determined in a TN20/20 luminometer (Turner Designs, Sunnyvale CA) in single tubes. To assure greater consistency in incubation times, we processed only four wells at a time.

For the time course experiment, we also determined the ATP concentration in the unattached (planktonic) bacteria present in the liquid culture medium that overlays the biofilm that forms on the bottom of the wells. 100 μl of liquid medium containing planktonic bacteria were removed from each well prior to the quantification of the biofilms. These were mixed with 100 μl of BacTitel-Glo™ reagent, incubated for 5 min, and measured in the TN20/20 luminometer.

CV assay

Biofilms were stained with 100 μl of 0.1% CV in H_2O at room temperature for 15 min. Following incubation, the CV solution was removed and the biofilms were washed twice with PBS. To elute bound CV, 100 μl of a mixture containing 80% ethanol and 20% acetone was added to each well and the plate was incubated at room temperature for 20 min. Finally, the mixture was diluted 1:20 with 80% ethanol/20% acetone and the optical density was determined at 600 nm.

Statistical analysis of the quantitative data

For each quantitative assay, the values obtained with the eight strains were tested using Analysis of Variance (ANOVA). A *p*-value below 0.05 indicates that at least one of the strains has a mean that differs from the others. For those assays that yielded a *p*-value below 0.05, Dunnett's test was performed as a post-hoc test. The significance level alpha was set at 0.05. This test determines whether the mean of a specific strain differs from the wild-type. Statistical analyses were performed on SAS v 9.1 (SAS Institute Inc., Cary NC).

Electron microscopy

Biofilms were grown on glass cover slips of 12 mm diameter (Assistant, Germany) in 6 well plates in 4 ml of TB, inoculated 1:100 from the overnight cultures. Biofilms were produced as described for the quantitative assays. The air-dried biofilms were fixed in 2 ml of 2.5% glutaraldehyde (Tousimis Research Corporation, Rockville MD) in 0.1 mol l^{-1} sodium phosphate buffer, rinsed once in the same buffer and then in deionized water. Biofilms were dehydrated, using a graded alcohol series (15 minutes each in 30%, 50%, 70%, and 90% ethanol in H_2O, twice for 15 min in 100% ethanol). Critical point drying was performed with an Autosamdri-810 critical point drier (Tousimis Research Corporation, Rockville MD) with liquid carbon dioxide as the transitional fluid. The cover slips were attached to aluminum mounts with adhesive carbon tabs or silver paint and coated with gold-palladium using a Balzers SCK 030 sputter coater (Balzers Union Ltd., Liechtenstein). Images were obtained with a JEOL JSM-6490LV scanning electron microscope (JEOL Ltd., Japan) at

3,000 ×, 6,500 ×, and 16,000 × magnification. Bacterial strains were processed two to five times; 8 to 26 images were obtained per strain. The distribution of bacteria across the cover slips was largely consistent. One representative image at 6,500 × is presented for each strain.

Results

In a previous technical report (Sule et al. 2008), we showed that bioluminescence correlates with both ATP concentration and bacterial cell number. In this report, we compare the ATP and CV assays for their utility in quantifying biofilm biomass.

To perform this comparison, we incubated bacteria of the wild-type strain (AJW678) and its isogenic *flhD*, *fimH*, *ompR*, *rcsB*, *rcsC*, *ackA*, and *ackA pta* mutants for 38 h. The ATP assay yielded three distinct classes (Fig. 1A): a wild-type-like class (which included the *flhD*, *rcsC*, *ackA*, and *ackA pta* mutants), a reduced signal class (which included only the *fimH* mutant), and an increased signal class (which included the *rcsB* and *ompR* mutants). In contrast, the CV assay yielded only two distinct classes of strains: a wild-type-like class and a reduced signal class that consisted of the *fimH* mutant. Statistical analysis with Dunnett's test confirmed the classification for both assays. Thus, both assays indicated that the amount of biomass attached to the bottom of the wells was considerably smaller for the *fimH* mutant than for its wild-type parent. In contrast, only the ATP assay indicated that the *ompR* and *rcsB* mutants produced significantly more biofilm-associated biomass than did their wild-type parent.

To validate this single time point experiment, we performed a time course experiment, limiting our study to a comparison between the biofilms formed by the *ompR* mutant and its wild-type parent (Fig. 1B). Whereas the peak ATP concentration of the *ompR* mutant was approximately the same amount as that of its wild-type parent, this peak occurred 12 h later, when the wild-type concentration was already in decline. Thus, at 38 h, the difference between the two strains was about two fold, which is consistent with the single time point experiment (Fig. 1A). To determine if the differences in biofilm-associated biomass could be attributed to differences in bacterial growth, we determined the ATP concentration in the planktonic bacteria present in the culture medium that overlays the biofilms. We found no significant difference in the ATP concentration (Fig. 1C). Thus, the differences in biofilm associated ATP concentrations (Fig. 1B) cannot be attributed to growth rate.

To test the results of the ATP assay, we used SEM to visualize 38 h biofilms produced by all the bacterial strains (Fig. 2). Consistent with both quantitative assays, fewer bacteria of the *fimH* mutant strain attached to glass. Consistent with the ATP assay (Fig. 1A), the *ompR* and *rcsB* mutants produced denser biofilms than did their wild-type parent. Consistent with both assays, the remaining strains produced an amount of biofilm quite similar to that of their wild-type parent. SEM also revealed substantial differences in the surface-associated structures exhibited by cells in biofilms formed by the different strains. Efforts to identify these structures are underway.

Discussion

In summary, the two quantitative assays were consistent with one another and the SEM for six out of the eight tested strains. Inconsistencies between the assays may have been due to the reliance of the assays on different modes of biomass determination. For example, the ATP assay detects live biomass in bacteria with low metabolic activity which makes it very suitable for physiological studies. In contrast, the CV assay detects both live and dead bacteria, as well as matrix. Thus, it may have limitations for studies that focus on the involvement of flagella or fimbriae in biofilm formation. For such studies, we recommend

the ATP assay. We highly recommend including time course experiments, especially for those mutants that differ from the wild-type in the single time point experiments. SEM appears to be a good qualitative control.

Taking the results from all three assays together, attachment appears to be the most critical step in the formation of biofilms. Type I fimbriae are the surface organelles that mediate irreversible attachment and the *fimH* mutant, which is unable to make the adhesive tip, had difficulties attaching to a surface and forming the biofilm. Flagella contribute to reversible attachment. This may explain why the *ompR* and *rcsB* mutants formed denser biofilms, as measured by the ATP assay and SEM. OmpR and RcsB are both two-component response regulators that, in their phosphorylated form, can bind to the *flhDC* promoter and shut down the expression of flagellar genes (Francez-Charlot et al. 2003; Shin and Park 1995). This means that mutants in *ompR* and *rcsB* exhibit increased flagellar synthesis relative to their wild-type parent (Fredericks et al. 2006; Oshima et al. 2002). For the *rcsB* mutant, this increase in the number of flagella appears to compensate for reduced levels of type I fimbriae (Schwan et al. 2007).

Abbreviations

ANOVA	Analysis of Variance
ATP	adenosine triphosphate
CV	crystal violet
LB	Luria Bertani broth
SEM	scanning electron microscopy
TB	tryptone broth
XTT	2,3-bis (methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide

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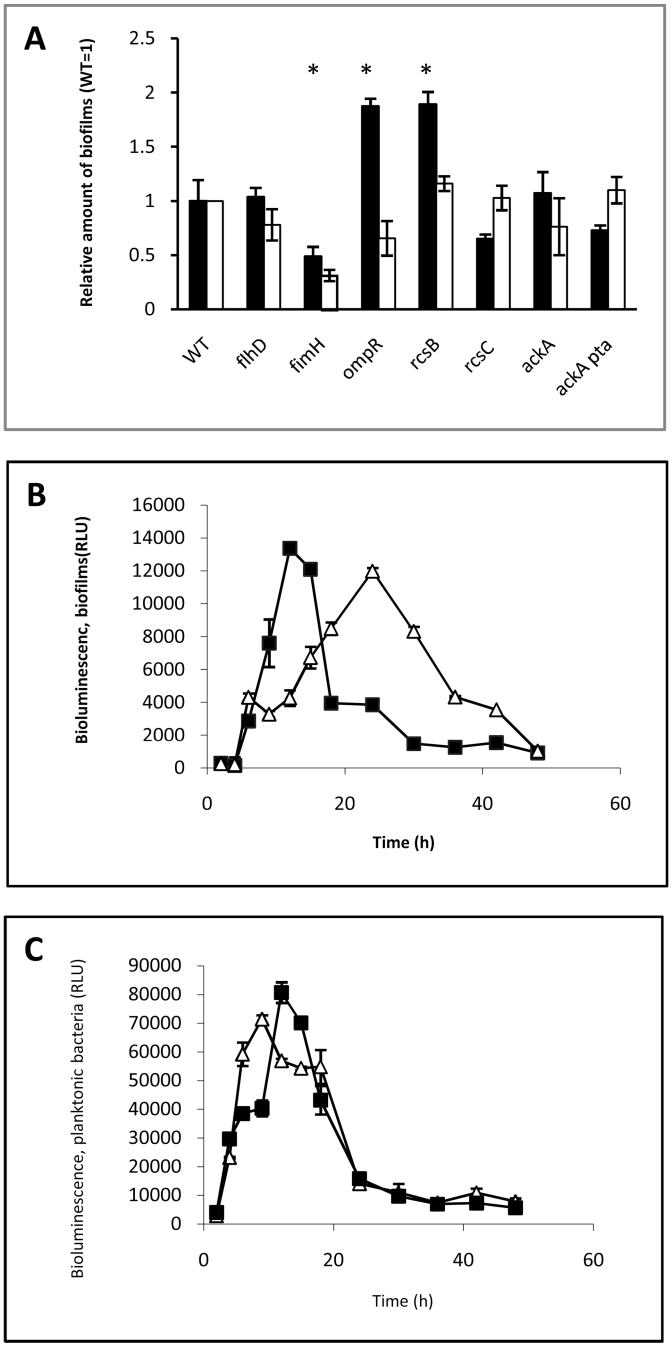

Figure 1.

Panel A describes the results of the quantitative assays. Biofilms were formed on polystyrene plates and the biomass was determined with the ATP (black bars) and the CV (white bars) assay. Averages are presented across three independent experiments, error bars indicate the standard deviation. Asterisks above the bars indicate strains that exhibited statistically significant differences from the wild-type strain, as determined by the ATP assay (according to Dunnett's test). Panel B is the time course of biofilm formation for the wild-type strain (closed squares) and the *ompR* mutant (open triangle). Panel C is the time course of ATP concentration in planktonic bacteria for the wild-type strain (closed squares) and the *ompR* mutant (open triangle).

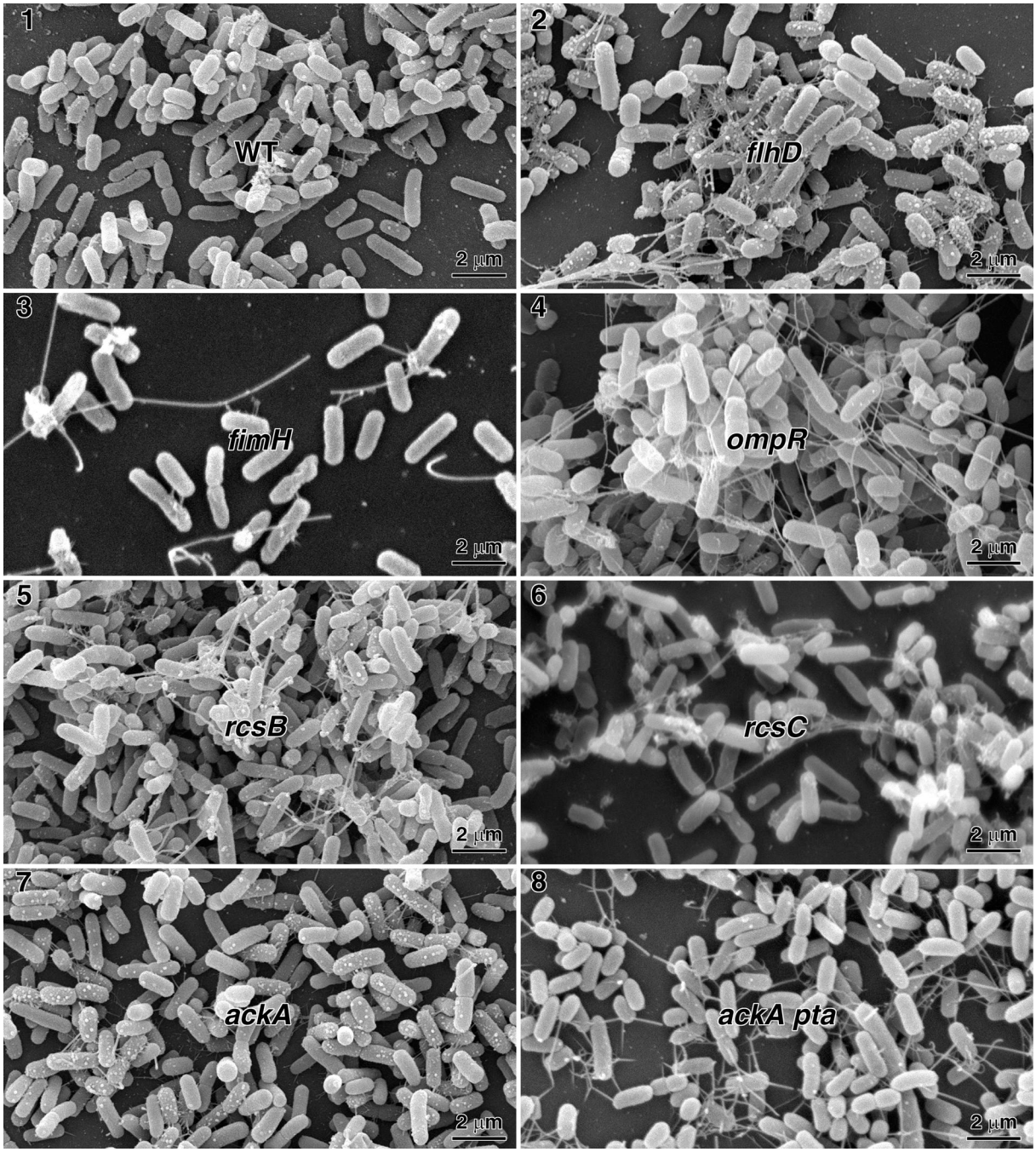

Figure 2.

Representative electron micrographs of biofilms formed by each of the strains at a magnification of 6,500 ×. A bar representing 2 μm is located within each image. Strains are as follows: 1, wild-type; 2, *flhD* mutant; 3, *fimH* mutant; 4, *ompR* mutant; 5, *rcsB* mutant; 6, *rcsC* mutant; 7, *ackA* mutant; 8, *ackA pta* mutant.