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A Combination of Phenotype MicroArray™ Technology with the ATP Assay Determines the Nutritional Dependence of *Escherichia coli* Biofilm Biomass

Preeti Sule, Shelley M. Horne and Birgit M. Prüß
North Dakota State University
USA

1. Introduction

Biofilms are defined as sessile communities of bacteria that form on surfaces and are entrapped in a matrix that they themselves produce. Biofilms cause severe problems 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), while being beneficial for waste water treatment and biofuel production (Wang and Chen, 2009). In addition, the bioremediation of crude oil spills involves a biofilm of oil degrading microbes, potentially supplemented by marine flagellates and ciliates (Gertler et al., 2010). Identifying the environmental conditions that prevent or support biofilm formation, as well as understanding the regulatory pathways that signal these conditions, is a pre-requisite to both, the solving of biofilm-associated problems and the use for beneficial purposes. In a previous study by our laboratory (Prüß et al., 2010), it was determined that nutrition ranked among the more important environmental factors affecting biofilm-associated biomass in *Escherichia coli* K-12. The key to this study was a high-throughput experiment, where biofilm biomass was determined in a collection of cell surface organelle and global regulator mutants under a variety of combinations of environmental conditions. The cell surface organelles each represented a distinct phase of biofilm formation (Sauer et al., 2002). Flagella are required for reversible attachment (phase I), curli or type I fimbriae are characteristic of irreversible attachment (phase II), and a polymeric capsule forms the matrix that permits the maturation of the biofilm (phase III). Eventually, flagellated bacteria are released from the biofilm (phase IV). Phases III and IV are particularly problematic for the disease progression. Bacteria that are located deep within the mature biofilm are particularly resistant to antibiotics and dispersed bacteria tend to serve as a reservoir that continuously feed the infection. Please, see Figure 1 for the distinction of biofilm phases.

The global regulators included in our previous study (Prüß et al., 2010) are involved in the co-ordinate expression and synthesis of biofilm-associated cell surface organelles. Many of them are components of two-component systems (2CSTS), each consisting of a histidine kinase and a response regulator (for reviews on 2CSTS signaling, please, see Galperin, 2004; Parkinson, 1993; West & Stock, 2001). In response to an environmental stimulus, the sensor kinase uses ATP as a phosphodonor to auto-phosphorylate at a conserved histidine, then

transferring the phosphate to the response regulator at a conserved aspartate residue. In addition, many response regulators can be phosphorylated in a kinase independent manner by the activated acetate intermediate acetyl phosphate (for a review on acetyl phosphate as a signaling molecule, please, see Wolfe, 2005). One 2CSTS that is involved in the formation of biofilms is EnvZ/OmpR, regulating the synthesis of flagella (Shin and Park, 1995), type I fimbriae (Oshima et al., 2002), and curli (Jubelin et al., 2005). RcsCDB is involved in the formation of biofilms, serving as an activator of colanic acid production (Gottesman et al., 1985). RcsCDB constitutes a rare phosphorelay, consisting of three proteins and four signaling domains (Appleby et al., 1996). Much of the effect of EnvZ/OmpR, and RcsCDB upon biofilm formation involves FlhD/FlhC (Prüß et al., 2006), which was initially described as a flagella master regulator (Bartlett et al., 1988) and later recognized as a global regulator of bacterial gene expression (Prüß & Matsumura, 1996; Prüß et al., 2001, 2003).

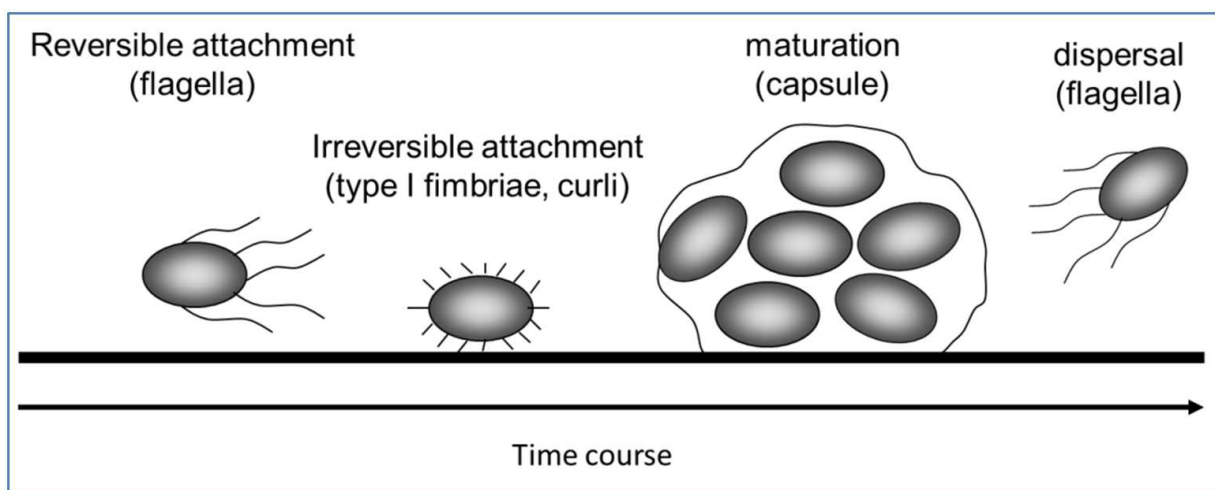


Fig. 1. Time course of biofilm formation

An early review article (Prüß et al., 2006) summarized the portion of the transcriptional network of regulation that centered around FlhD/FlhC. This partial network contained 16 global regulators, among them many 2CSTSs, such as EnvZ/OmpR, RcsCDB, and CpxR. The regulation of approximately 800 genes was affected by the network. Since many of these encoded components of the biofilm-associated cell surface organelles, it was hypothesized that the network may affect biofilm formation. This hypothesis was confirmed by the high-throughput study that led to the identification of nutrition as one of the more instrumental factors in determining biofilm biomass (Prüß et al., 2010). The global regulators that were part of the network led to the mutant collection for the experiment. Among the tested environmental conditions were temperature, nutrition, inoculation density, and incubation time. Temperature and nutrition were more important in determining biofilm biomass than were inoculation density and incubation time. The mutant screen was consistent with the idea that acetate metabolism may act as a nutritional sensor, relaying information about the environment to the development of biofilms. This hypothesis was confirmed by scanning electron microscopy. A new 2CSTS, DcuS/DcuR, was identified as important in determining the amount of biofilm-associated biomass (Prüß et al., 2010).

The high-throughput experiment merely determined that nutrient rich bacterial growth media are more supportive of biofilm formation than are nutrient poor media. Specific nutrients that are supportive or inhibitory to biofilm formation were not determined and are

the next logical step. This will be dependent on an assay system that quantifies biofilm biomass in the presence of an array of single nutrients. With this study, we will introduce such a system that quantifies biofilm biomass formed by *Escherichia coli* mutants in the presence of single nutrients by combining the Phenotype MicroArray™ technology from BioLog (Hayward, CA) with the ATP quantitative biofilm assay that was previously developed by our own lab (Sule et al., 2009), followed up by statistical analysis of the data, and metabolic modeling.

The BioLog Phenotype MicroArray (PM) technology has been developed for the determination of bacterial growth phenotypes (Bochner, 2009; Bochner et al., 2001, 2008). The PM technology consists of 96 well plates with 95 single nutrients dried to the base of each of 95 wells (the additional well constitutes the negative control). When used with the tetrazolium dye that is provided by the manufacturer and indicative of respiration, the PM system is used to determine growth of bacterial strains on single nutrients. Since the total system consists of 20 of such plates, the user is enabled to screen growth under close to 2,000 conditions. The plates are designated PM1 through PM20, with PM1 and PM2 containing carbon sources, PM3 containing nitrogen sources, and PM4 containing sulfur and phosphorous sources. The remaining plates can be used to determine the pH range of growth or resistance to antibiotics or other harsh conditions. Liquid growth media are supplied together with the respective plates.

With respect to bacterial growth, PMs have been used in numerous previous studies (Baba et al., 2008; Edwards et al., 2009; Mascher et al., 2007; Mukherjee et al., 2008; Zhou et al., 2003). However, use of this technology for the investigation of biofilms has been limited (Boehm et al., 2009). In *E. coli*, the use of PM technology for the quantification of biofilm biomass has not been reported. In addition, the previous use of PM technology in biofilm studies has been based on the use of the crystal violet assay for the quantification of biomass. There are, however, many more assays that have been developed for the quantification of biofilm-associated biomass, each of which serves a different purpose. The different quantitative biofilm assays are compared in Table 1.

Crystal violet is a non-specific protein dye that stains the bacterial cells and their exopolysaccharide matrix for dead and live bacteria alike. Biofilms are cultivated on 96 well plates and stained with 0.1% crystal violet in H₂O. In a second step, crystal violet is solubilized with a mix of ethanol and acetone (80:20) and measured spectrophotometrically (O'Toole et al., 1999; Pratt & Kolter, 1998). The assay was developed as a high-throughput assay that is suitable for robotic instrumentation (Kugel et al., 2009; Stafslie et al., 2006, 2007). ATP (adenosine triphosphate) (Sule et al., 2008, 2009) and XTT (4-nitro-5-sulfophenyl-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Cerca et al., 2005) are both assays that quantify the energy metabolism of the bacteria. Therefore, only biomass of live bacteria is considered. ATP is converted by the enzyme luciferase into a bioluminescence signal, XTT is reduced by NADH to an orange colored water-soluble formazan derivative. Similar to crystal violet, fluoro-conjugated lectins quantify the biomass of live and dead bacteria alike (Burton et al., 2006). Lectins are highly-specific carbohydrate binding proteins that have been utilized to quantify different cell wall components, as well as extracellular matrix (Stoitsova et al., 2004). Specifically, wheat germ agglutinin (WGA) and soybean agglutinin (SBA) selectively complex lipooligosaccharides and colanic acid, respectively. For our experiments, we needed an assay that quantifies biofilm biomass in live bacteria that is also suitable for high-throughput experimentation, cost effective, and rapid. The ATP assay appeared as the most suitable assay among the five compared assays (Table 1).

Assay	Live/dead cells	Detected material	High-throughput suitability	Reference
Crystal violet	Live and dead cells	Exopolysaccharide	Yes	(Kugel et al., 2009; Stafslie et al., 2006, 2007)
ATP	Live cells	Energy (ATP)	Yes	(Sule et al., 2008, 2009)
XTT	Live cells	Energy (NADH)	Yes	(Cerca et al., 2005)
WGA	Live and dead cells	Lipooligosaccharide	Not tested	(Burton et al., 2006; Stoitsova et al., 2004)
SBA	Live and dead cells	Colanic acid	Not tested	(Burton et al., 2006; Stoitsova et al., 2004)

Table 1. Comparison of different quantitative biofilm assays

In the past, ATP has been used as a measure of biomass (Monzón et al., 2001; Romanova et al., 2007; Takahashi et al., 2007) because its concentration is relatively constant across many growth conditions (Schneider & Gourse, 2004). For the quantification of biofilms, the BacTiter Glo™ assay from Promega (Madison WI) has been used for biomass determination in *Pseudomonas aeruginosa* (Junker & Clardy, 2007) and *E. coli* (Sule et al., 2008, 2009). In *E. coli*, we established that a two fold increase in bioluminescence did indeed relate to a two fold increase in the ATP concentration and a 2 fold increase in the number of bacteria (Sule et al., 2008). Across eight isogenic *E. coli* strains (one parent strain and seven mutants), differences in biofilm biomass that were determined with the ATP assay were paralleled by observations made with scanning electron microscopy (Sule et al., 2009).

The protocol involves the formation of the biofilms on 96 well micro titer plates, incubation at the desired temperature, and washing of the biofilms with phosphate buffered saline (PBS). Special attention is needed to distinguish the pellicle that forms at the air-liquid interface from the biofilm that forms at the bottom of the wells. In particular, the AJW678 derivatives that we are working with form a solid pellicle that covers the entire surface of the culture (Wolfe et al., 2003). For users who like to include the pellicle into their study, the growth medium and the PBS will be pipetted off carefully from each well. Users who wish to discard of the pellicle can flip the entire 96 well plate over and remove the liquid this way. Eventually, 100 µl of BacTiter Glo reagent are added to each well. After 5 min of incubation, bioluminescence is measured.

For this study, we will use the ATP assay to quantify biofilm biomass that forms on the PM1 plate of BioLog's PM system. The PM1 plate contains 95 single carbon sources in addition to the negative control. Besides the fact that the use of PM technology for the determination of the nutritional requirements of biofilm has not been reported in *E. coli* yet, the combination of PM technology with the ATP assay is novel. The combination of both, PM technology and ATP assay, together with the statistical analysis and metabolic modeling, enables the rapid screening of thousands of nutrients for their ability to support or inhibit growth and biofilm formation in one experimental setup. The described technique is not only cost-efficient and easy to perform, but also high-throughput in nature, providing valuable insight into the nutritional requirements during biofilm formation.

2. Materials and methods

2.1 Bacterial strains and growth conditions

The bacterial strains used in this study were the *E. coli* parental strain AJW678, which was characterized as an efficient biofilm former (Kumari et al., 2000) and its isogenic *flhD*, *fliA*, *fimA*, and *fimH* mutants. The *flhD* mutant was constructed by P1 transduction, using MC1000 *flhD::kan* (Malakooti, 1989) as a donor and AJW678 as a recipient. This resulted in strain BP1094. AJW2145 contained a *fliA::Tn5* insertion, AJW2063 a *fimA::Kn* mutation, and AJW2061 a *fimH::kn* mutation, all in AJW678 (Wolfe et al., 2003). The mutations abolish expression of FlhD/FlhC, FliA, FimA, and FimH, respectively. As a consequence, mutants in *flhD* and *fliA* are non-motile, whereas mutants in *fimA* are lacking the major structural subunit and mutants in *fimH* the mannose specific adhesive tip of the type I fimbrium. Bacterial strains were stored at -80°C in 8% dimethylsulfoxide, plated onto Luria Bertani plates (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) prior to use, and incubated overnight at 37°C. Bacterial strains are summarized in Table 2.

Strain	Relevant genotype	Reference
AJW678	<i>thi-1 thr-1(am) leuB6 metF159(am) rpsL136 ΔlacX74</i>	(Kumari et al., 2000)
BP1094	AJW678 <i>flhD::kn</i>	(Prüß et al., 2010)
AJW2145	AJW678 <i>fliA::Tn5</i>	(Wolfe et al., 2003)
AJW2063	AJW678 <i>ΔfimA::kn</i>	(Wolfe et al., 2003)
AJW2061	AJW678 <i>fimH::kn</i>	(Wolfe et al., 2003)

Table 2. Bacterial strains used for this study

2.2 Strain selection for the biofilm experiment

For this study, a mutation was needed that would abolish one of the early cell surface organelles that contribute to the biofilm, while still permitting the formation of biofilms. We performed scanning electron microscopy (SEM) to determine the ability of the five bacterial strains (parental strain, *flhD* mutant, *fliA* mutant, *fimA* mutant, *fimH* mutant) to form biofilms. Biofilms were grown for 38 h at 37°C on glass cover slips with tryptone broth (TB; 1% tryptone, 0.5% NaCl) as a growth medium. Biofilms were fixed in 2.5% glutaraldehyde and prepared for SEM as described (Sule et al., 2009). Images were obtained with a JEOL JSM-6490 LV scanning electron microscopy (SEOL Ltd., Tokyo, Japan) at 3,000 fold magnification. 10 to 15 images were obtained per bacterial strain from at least three independent biological samples. One representative image is shown per bacterial strain.

2.3 Biofilm quantification with PM technology and the ATP assay

We used the PM1 plate of the BioLog PM system that contains 95 single carbon sources. When used with the tetrazolium dye that is provided by the manufacturer and indicative of respiration (Bochner et al., 2001), the PM system can be used for measuring growth of bacterial strains on single nutrients. We here describe a protocol for the determination of biofilm amounts (Figure 2).

As recommended by the manufacturer for the determination of growth phenotypes, the bacterial cultures were streaked from LB plates onto R2A plates (to deplete nutrient stores) and incubated at 37°C for 48 hours. Bacteria were removed from the plates with a flocced

swab (Copan, Murrieta CA), resuspended and then further diluted with IF-0a GN/GP Base (BioLog, Hayward CA) inoculation fluid to an optical density (OD_{600}) of 0.1. Leucine, methionine, threonine and thiamine were added at a final concentration of 20 $\mu\text{g}/\text{ml}$, the redox dye that is used for the determination of growth phenotypes was omitted for biofilm quantification. 100 μl of the inoculum was then dispensed into each of the 96 wells of the PM1 plates. The inoculated plates were wrapped with parafilm to minimize evaporation and incubated at 37°C for 48 hours. Biofilm amounts were quantified using the previously described ATP based technique (Sule et al., 2008, 2009). Briefly, the growth medium was carefully aspirated out of each well, minimizing loss of biofilm at the air liquid interface. The biofilms were then washed twice with phosphate buffered saline (PBS) in order to remove any residual media components. The biofilms were air dried and quantified using 100 μl BacTiter Glo™ reagent (Promega, Wisconsin, WI). The biofilms were incubated with the reagent for 10 min at room temperature and the bioluminescence was recorded using a TD 20/20 luminometer from Turner Design (Sunnyvale, CA). The bioluminescence was reported as relative lux units (RLU).

The determination of biofilm amounts in the presence of single nutrients was performed four times for each strain. In addition, growth on these carbon sources was determined in three independent replicate experiments, following the protocol that is described for the determination of growth phenotypes and including the redox dye (Bochner et al., 2001). Carbon sources on which both strains grew to an average OD_{600} of 0.5 or more were selected for the *t*-test analysis and carbon sources on which each strain grew to an average OD_{600} of 0.5 or more were selected for the ANOVA/Duncan analysis of biofilm amounts (see below).

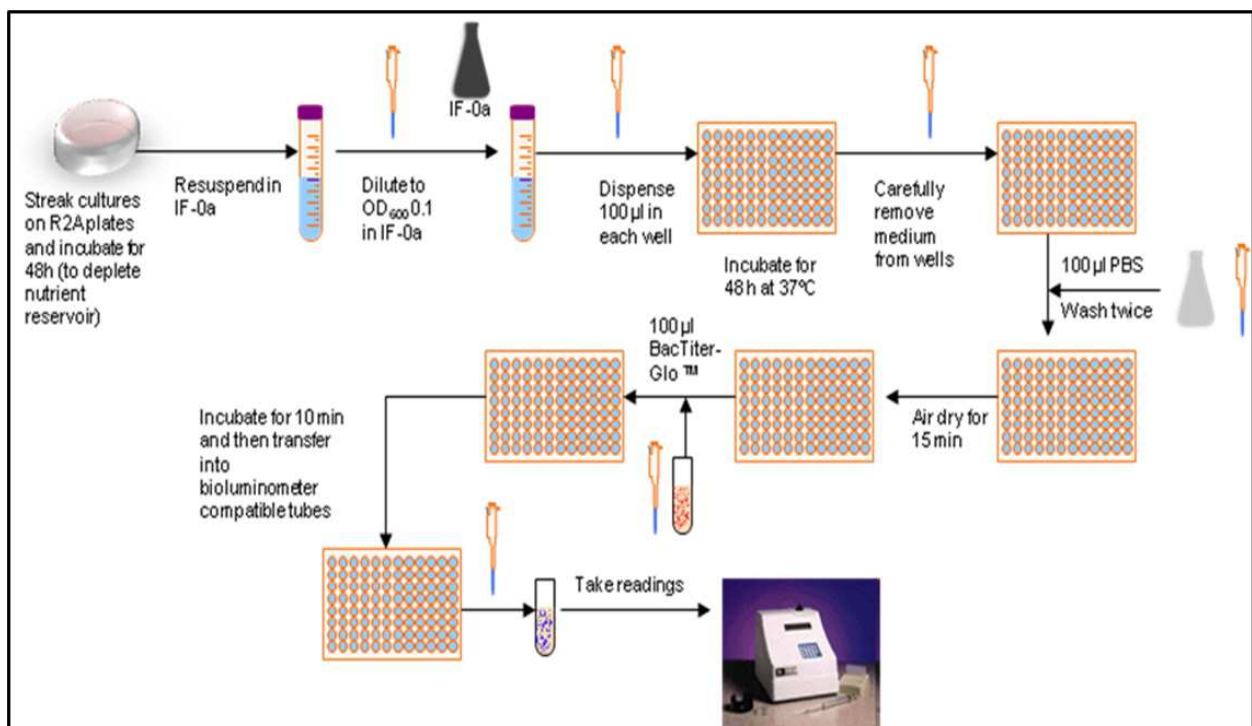


Fig. 2. Work flow for the determination of biofilm amounts on PM plates with the ATP assay

2.4 Data analysis

Prior to the statistical analysis, the biofilm amounts from each strain were normalized for experiment specific variation; total bioluminescence across each experiment was summed

up and the fold variation was calculated, using the lowest experiment as a norm (1 fold). Data points in each experiment were divided by the respective fold variation. The normalized experimental data sets were subjected to two independent types of statistical analysis, all done using SAS software (SAS Institute Inc., 2009). First, we performed Student's *t*-test on all those carbon sources on which both strains grew to an average OD₆₀₀ of 0.5 or more to determine statistically significant differences between the amounts of biofilm that were formed on a given carbon source between the two strains. Since this analysis yielded more carbon sources than we could comprehend on a physiological level, we then analyzed each strain individually and then compared biofilm amounts on individual carbon sources for specific nutrient categories of structurally related carbon sources. For this analysis, the normalized biofilm data from each strain were subjected to separate one way ANOVAs, followed up with Duncan's multiple range tests. The tests compared the means of the amount of biofilm formed in the presence of each carbon source to all the other carbon sources within each strain. Carbon sources whose mean was different from the means of all the other carbon sources with statistical significance formed their own group in the Duncan's test. Carbon sources whose mean difference from the other carbon sources was not statistically significant formed overlapping groups.

Performing Duncan's test on the parent strain, two carbon sources formed groups A and B. Among the remaining carbon sources, we determined those that were structurally related to group A and B carbon sources. This was done after a determination of the respective chemical structures with the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa & Goto, 2000; KEGG, 2006). Biofilm amounts formed by the *flhD* mutant were compared to the parent strain for all these carbon sources. In a second analysis, one carbon source formed group A in the Duncan's test for the *flhD* mutant. Among the remaining carbon sources, we identified two carbon sources that were structurally related. Biofilm amounts for these three carbon sources were compared between the two strains. For both analyses, data were summarized in a Table (3 and 4).

2.5 Metabolic modeling

Metabolic pathways that lead to the degradation of all the carbon sources that are discussed in this study were determined with KEGG. Metabolic intermediates that were common between different pathways were used to construct metabolic maps. Pathways for both strains were combined in Figures 5 and 6.

3. Results

3.1 Strain selection using electron micrographs

To determine the ability to form biofilm, electron microscopy was performed with the five strains that were listed in Materials and Methods. Figure 3 depicts one representative illustration of the 10 to 15 images that were obtained per bacterial strain. Most of these strains formed biofilm despite mutations affecting cell surface organelles of either reversible (flagella) or irreversible (type I fimbriae) attachment. The sole exception was the *fimH* mutant which only showed a small number of scattered bacteria attached across the slide. The *fimA* mutant exhibited a large number of filamentous appendages. We are currently unable to explain these appendages.

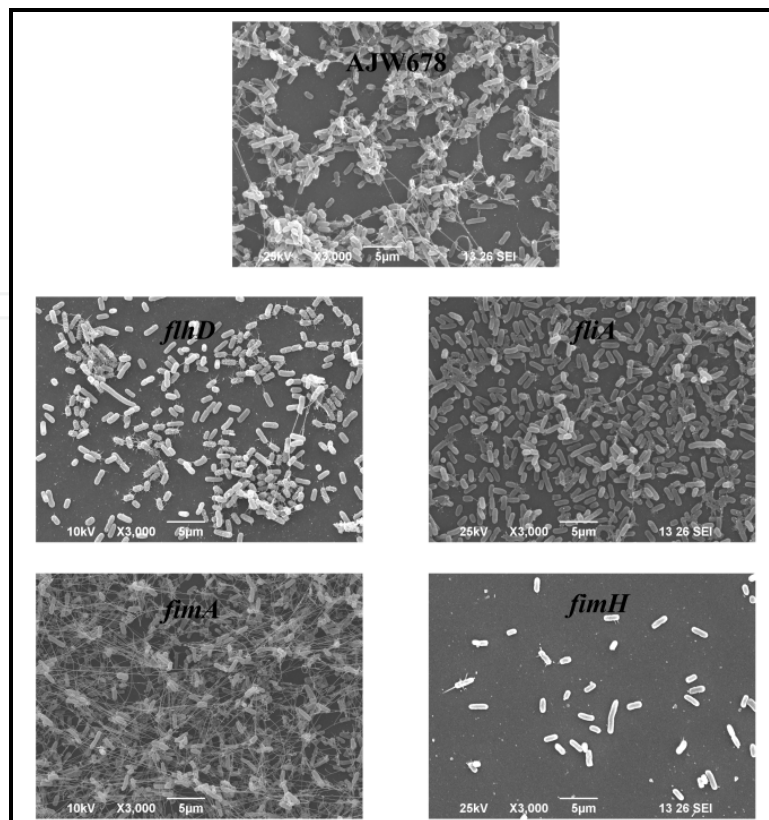


Fig. 3. Electron micrographs at 3,000 fold magnification for the AJW678 parent strain, and its isogenic mutants in *flhD*, *fliA*, *fimA*, and *fimH*

We wanted a strain for the phenotype microarray experiment that was able to form biofilm on complex media, while lacking one of the cell surface organelles. Since the amount of biofilm formed by the *flhD* mutant was similar to that of the parental strain in the electron micrographs, the *flhD* mutant was selected for further testing using the PM1 plates. The *flhD* mutant has as an additional advantage that much of the regulation by FlhD/FlhC has been previously described. This vast amount of information will help us to analyze the complex metabolic data.

3.2 Biofilm quantification with PM technology and statistical analysis

Biofilms that formed on the PM1 plates were quantified with the ATP assay and compared between the two strains with the *t*-test. The analysis did not yield any carbon sources that supported more biofilm in the parent strain than in the mutant. The 25 carbon sources that yielded significantly higher amounts of biofilm in the *flhD* mutant are demonstrated in Figure 4. Since the carbon sources that supported biofilm formation by the mutant more so than by the parent are numerous, we decided to analyze each strain statistically first and focus the comparison between the strains to specific structural categories of carbon sources. These are designated 'nutrient categories' throughout this manuscript.

3.2.1 Carbon sources that formed their own duncan's group for the parent strain

The normalized data set from the parent strain was subjected to Duncan's multiple range test. According to this test, the two carbon sources that were the best biofilm supporters for the parent *E. coli* strain, maltotriose and maltose, formed exclusive groups A and B. Without

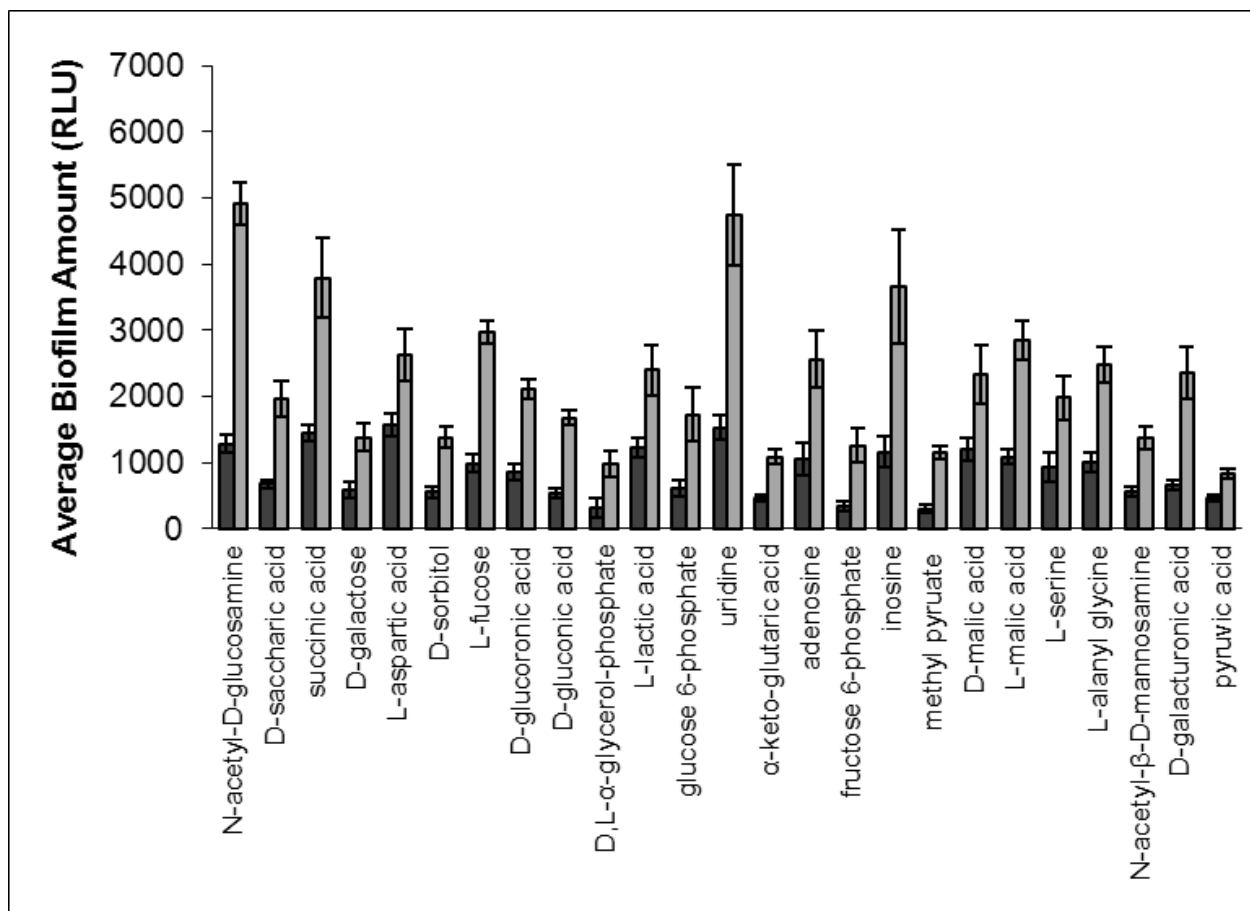


Fig. 4. Biofilm formation in the parent strain and the *flhD* mutant were compared using a *t*-test. The dark shaded bars resemble the parent strain, the lighter bars the mutant. The error bars in the graph indicate the standard deviation. Note that only carbon sources were included in this analysis that supported growth to at least 0.5 OD₆₀₀ in both strains.

forming its own Duncan group, ribose was the carbon source that supported the smallest amount of biofilm among all carbon sources tested, while still supporting growth. The parent strain also formed good amounts of biofilm on the remaining C6-sugars. Interestingly, the amount of biofilm that formed on maltotriose (trisaccharide of glucose) was roughly three times the amount of biofilm that formed on glucose. The amount of biofilm that formed on maltose (disaccharide of glucose) was about twice the amount that formed on glucose. The C5-sugars xylose and lyxose did not support growth of the parental strain to the cutoff of 0.5 OD₆₀₀. For all these carbon sources, biofilm amounts formed by the *flhD* mutant were compared to the parent strain (Table 3). In contrast to the parental strain, the *flhD* mutant did not grow well on C6-sugars and their oligosaccharides. Unlike the parental strain, the mutant did not grow well on ribose, but grew to the cut off of 0.5 OD₆₀₀ on lyxose and xylose. Still, the amount of biofilm formed by this strain on C5-sugars was low (<1,000 RLU). An interesting phenomenon was observed for sugar phosphates and sugar acids. Sugar phosphates supported biofilm production by the mutant more so (>1,200 RLU) than for the parent strain (<600 RLU). Likewise, sugar acids were found to be good supporters of biofilm for the *flhD* mutant strain (1,500 to 2,500 RLU), but not for the parent (500 to 800 RLU). This was even more remarkable, considering the fact that the parental strain (OD₆₀₀ ~ 1.0) grew better on sugar acids than the *flhD* mutant (OD₆₀₀ of 0.2 to 0.8).

Nutrient category	Nutrients	AJW678	<i>flhD</i> mutant
		Biofilm Amount (RLU)	Biofilm Amount (RLU)
Trisaccharide	Maltotriose	4,935	NA*
Disaccharide	Maltose	2,928	NA*
C6-sugars	Glucose	1,615	NA*
	Fructose	1,500	NA*
	Mannose	1,745	NA*
	Rhamnose	873	NA*
C5-sugars	Ribose	147	NA*
	Lyxose	NA	650
	Xylose	NA	544
Sugar phosphates	Glucose 6-P	614	1,722
	Fructose 6-P	338	1,258
Sugar acids	D-galacturonic acid	668	2,358
	D-gluconic acid	532	1,679
	D-glucuronic acid	852	2,110

Table 3. Biofilm amounts on carbon sources which formed their own Duncan's grouping for the parent strain and structurally related carbon sources. Columns 1 and 2 indicate the nutrient categories and single carbon sources for which data are included. Columns 3 and 4 represent biofilm amounts for the parent strain and the mutant on carbon sources that permitted growth to more than 0.5 OD₆₀₀. NA denotes 'not applicable', where the strain grew to an OD₆₀₀ below 0.5.

3.2.2 Carbon source that formed its own duncan's group for the *flhD* mutant

The amount of biofilm formed on each carbon source by the *flhD* mutant was quantified and subjected to Duncan's multiple range test. According to the Duncan's grouping, the sole carbon source that formed its own group A for the *flhD* mutant was N-acetyl-D-glucosamine. Structurally related carbon sources that were included in the PM1 plate are D-glucosaminic acid and N-acetyl-β-D-mannosamine. Biofilm amounts formed on these three carbon sources were compared between the two strains (Table 4).

Nutrient category	Nutrients	<i>flhD</i> mutant	AJW678
		Biofilm Amount (RLU)	Biofilm Amount (RLU)
Sugar amines	N-acetyl-D-glucosamine	4,911	1,285
	D-glucosaminic acid	660	NA
	N-acetyl-β-D-mannosamine	1,368	559

Table 4. Biofilm amounts on carbon sources which formed their own Duncan's grouping for the *flhD* strain and structurally related carbon sources. Columns 1 and 2 indicate the nutrient category and single carbon sources for which data are included. Columns 3 and 4 represent biofilm amounts for the *flhD* mutant and its parent strain on carbon sources that permitted growth to more than 0.5 OD₆₀₀. NA denotes 'not applicable', where the strain grew to an OD₆₀₀ below 0.5.

On N-acetyl-D-glucosamine, the *flhD* mutant (4,900 RLU) formed a significantly larger amount of biofilm than the parent strain (1,300 RLU), while both strains grew to approximately 1 OD₆₀₀. On D-glucosaminic acid, the parent strain did not grow to the cutoff OD of 0.5. The *flhD* mutant grew well, but the amount of biofilm biomass was poor (~600 RLU). For N-acetyl-β-D-mannosamine, both strains grew well, the *flhD* mutant expressed more than twice the ability to form biofilm than its isogenic parent.

3.3 Metabolic modeling

Metabolic pathways were drawn for the degradation of all those carbon sources that supported amounts of biofilm larger than 1,000 RLU for one of the tested strains. These are carbon sources of the nutrient categories C6-sugars, sugar phosphates, sugar acids, and sugar amines. C6-sugars all have pathways that feed into the Embden-Meyerhof pathway, sugar phosphates are intermediates of this pathway. As shown in Figure 5, mannose, fructose, and N-acetyl D-glucosamine feed into fructose 6-phosphate. Gluconate, glucuronate, galacturonate, and rhamnose feed into glyceraldehyde 3-phosphate. This leads to the production of acetyl-CoA, acetyl phosphate and acetate (Figure 6).

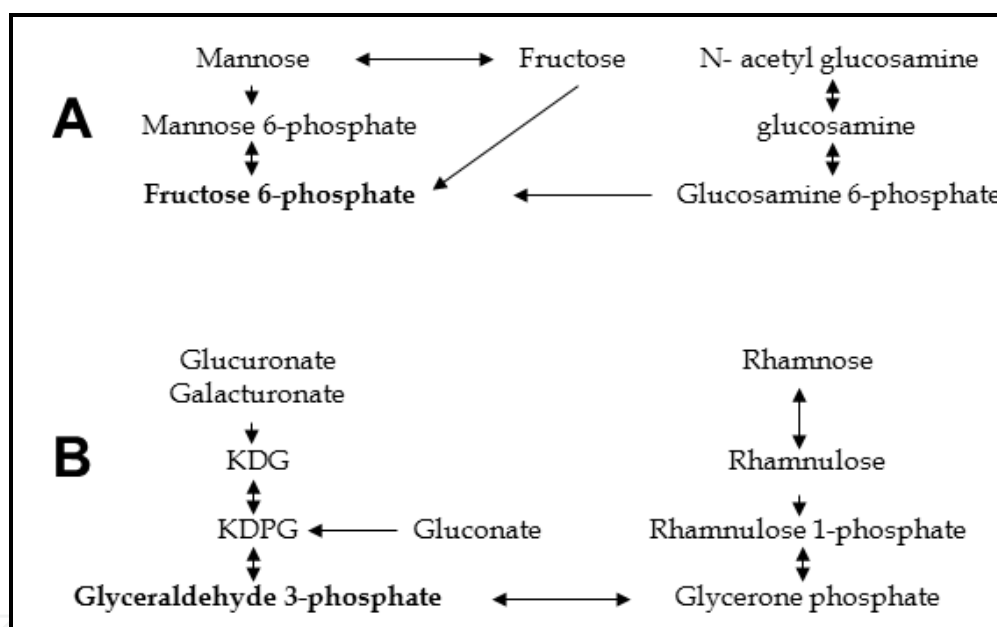


Fig. 5. Metabolic pathways from the top biofilm producing carbon sources for both *E. coli* strains, feeding into the Embden-Meyerhof pathway.

4. Discussion

4.1 Development of the combination assay

Altogether, we present an assay that builds upon two previous assays, the PM technology and the ATP assay. Both assays have been used in much different contexts previously. PM plates have been commonly used to discover various bacterial characteristics based on phenotypic changes (Bochner et al., 2008). Studies involving PM plates include the evaluation of the alkaline stress response induced changes in the metabolism of *Desulfovibrio vulgaris* (Stolyar et al., 2007). PMs have also been used for the identification of bacterial species (Al-Khalidi & Mossoba, 2004). The use of PM technology in biofilm research is

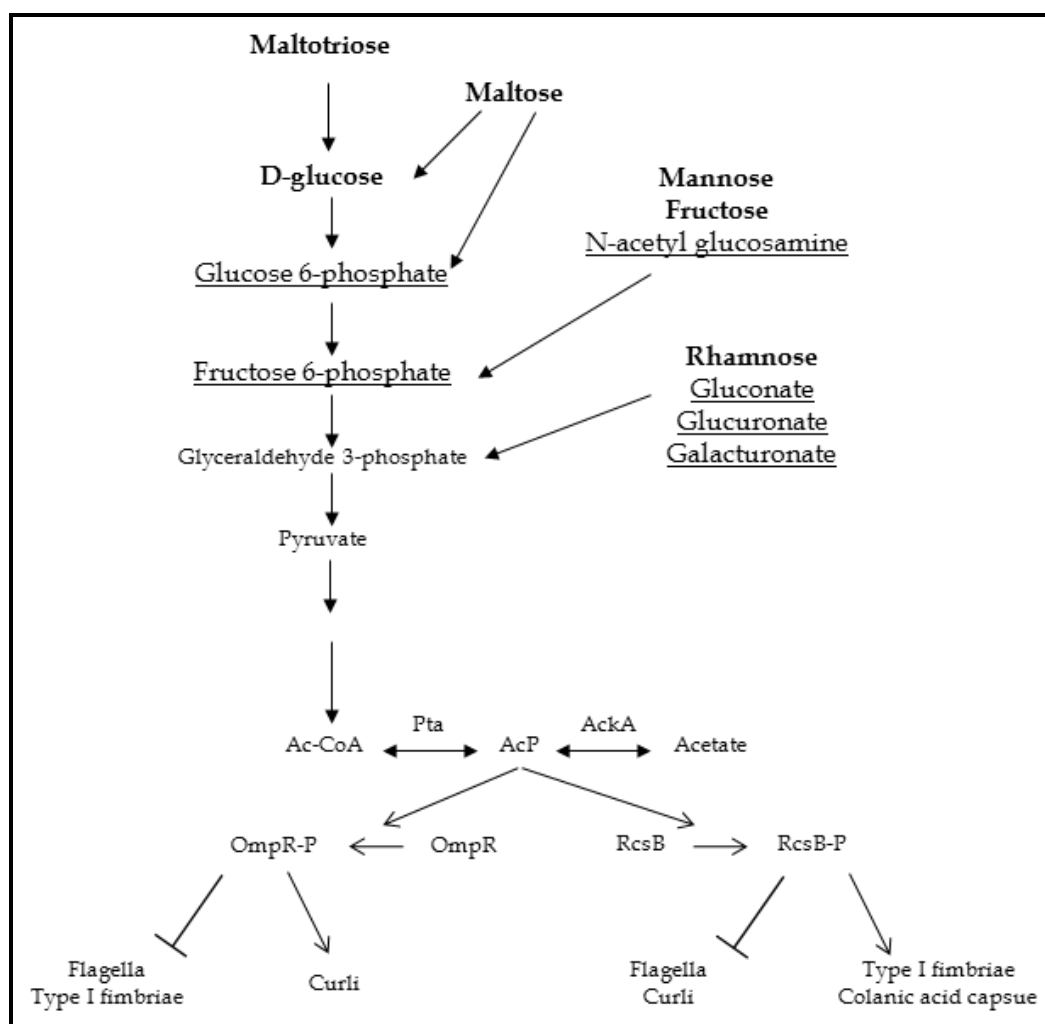


Fig. 6. Metabolic pathways from the top biofilm producing carbon sources for both strains to the production of acetate. Carbon sources that are printed in bold were top biofilm supporters for the parent strain. Carbon sources that are underlined were top biofilm supporters for the *flhD* mutant. The effect of acetyl phosphate on RcsB and OmpR on the synthesis of flagella, curli, fimbriae, and capsule is indicated.

limited to a study of the ability of *E. coli* to form biofilm upon ribosomal stress (Boehm et al., 2009). That study used the crystal violet assay as a detection tool for the amount of biofilm. Here we report for the first time a combination of the established ATP assay along with the PM technology to assess nutritional dependence of *E. coli* during biofilm formation. Since the statistics approach alone (*t*-test) yielded no more than a list of data that were difficult to interpret, we decided for a combined statistics/metabolism approach to analyze the complex data. The combination of the two experimental parts of the assay together with the two analysis parts enables the user to rapidly screen hundreds and thousands of single nutrients for their ability to inhibit growth and biofilm formation in one experimental setup. Integrating different mutants into the study will yield valuable insight into the regulatory mechanisms that are involved in the signaling of these nutrients. The described technique is not only cost-efficient and easy to perform, but also high-throughput in nature. It is ideally suited to provide valuable insight into the nutritional requirements that determine biofilm biomass, as well as the respective signaling pathways.

4.2 Biological analysis of the data

In the described study, we observed that the FlhD mutants made quantitatively higher amounts of biofilms on numerous carbon sources. Interestingly, the parental strain did not form higher quantities of biofilm than the mutant on any of the tested carbon sources. These observations shed light into the ongoing controversial debate, elucidating the role of motility in biofilm formation. In certain bacterial species including *Yersinia enterocolitica*, the presence of motility has been shown to be beneficial for biofilm formation (Wang et al., 2007). Several previous studies from our lab demonstrate that the absence of motility enhances the ability of *E. coli* to form substantial amounts of biofilm. As one example, strains transformed with the FlhD expressing plasmid pXL27 showed diminished biofilm forming capabilities (Prüß et al., 2010). Additionally, ongoing studies carried out in the lab with *E. coli* O157:H7 and the *E. coli* K-12 strains MC1000 and AJW678 point in the same direction, exemplifying our belief that FlhD and motility are detrimental to biofilm formation for our bacterial strains and under the conditions of our experiments (Sule et al., unpublished data). As a second observation, carbon sources that supported maximal biofilm formation by either strain all fed into glycolysis eventually, and produced acetate. Although the carbon sources that promoted the highest biofilm amounts were different for the two strains, they still were in the same pathway. The previous high-throughput experiment that had pointed towards nutrition as instrumental in determining biofilm associated biomass had also postulated acetate metabolism as one of the key players in biofilm formation (Prüß et al., 2010). Phosphorylation of OmpR and RcsB by the activated acetate intermediate acetyl phosphate (Kenney et al., 1995) and acetylation of RcsB by acetyl-CoA (Thao et al., 2010) have been described in the past. These activated 2CSTS response regulators then affect the expression level of biofilm associated cell surface organelles, such as flagella, type I fimbriae, curli, and capsule (Ferrieres & Clarke, 2003; Francez-Charlot et al., 2003; Oshima et al., 2002; Prüß, 1998; Shin & Park, 1995) (Figure 6). The positive effect on biofilm amounts of carbon sources that lead to the production of acetate can be explained with the combined inhibitory effect of acetyl phosphate and acetyl-CoA on flagella through OmpR and RcsB and the above described disadvantage of flagella and motility during biofilm formation. We however do not state that acetate is the sole controlling mechanism as the complexity of the bacterial system cannot be explained based on a small number of signaling molecules. The most striking observation obtained from our studies pertains to the pattern of growth and biofilm formation on sugar acids. It was observed that the FlhD mutants grew to lower optical densities on sugar acids, but formed much higher amounts of biofilm as compared to the parental strain. Previous work from the Prüß lab had shown similar defects in growth of *flhD* mutants on sugar acids (Prüß et al., 2003), biofilm formation was not tested in that study. The inverse effect of sugar acids on growth and biofilm amounts may have implications in the intestine. Mutants in *flhD* have an early disadvantage in colonization, but recover after prolonged incubation (Horne et al., 2009). They even take over the population after more than two weeks (Leatham et al., 2005). The initial lack of colonization could be explained by the inability of the *flhD* mutant to degrade the numerous sugar acids present in the intestine (Peekhaus & Conway, 1998). On the other hand, the ability to take over the bacterial population at a later stage may have to do with the lack of the flagellin, which is a potent cytokine inducer (McDermott et al., 2000). The here discovered ability to make an increased amount of biofilm may add to the long term survival of *flhD* mutants in the intestine. Bacteria deep within the biofilm will be protected from the immune system, while metabolizing very slowly and not needing much nutrition.

Among the carbon sources that were the least supportive of biofilm formation, the inability of the C5-sugars to support growth and/or biofilm formation was the most striking. Ribose supported growth by the parent strain, but yielded the lowest biofilm amount of all tested carbon sources. The *flhD* mutant did not even grow on ribose. According to Fabich and coworkers (Fabich et al., 2008), ribose is not among the carbon sources that the *E. coli* K-12 strain MG1655 utilizes when bacteria colonize the intestine. Our data are consistent with this observation. Since *E. coli* O157:H7 EDL933 does actually utilize ribose in the intestine, ribose utilization may constitute a mechanism by which pathogenic *E. coli* can find a niche in the intestine to co-exist with the commensal *E. coli* strains.

The inability to grow on lyxose is also consistent with previous observations, where only a mutation in the *rha* locus enabled the bacteria to grow on lyxose via the rhamnase pathway (Badia et al., 1991). Normally, *E. coli* are unable to grow on lyxose. Most interesting is the behavior of the two strains on xylose. The parent *E. coli* strain was unable to grow on xylose. The *flhD* mutant did grow, while producing moderately low amounts of biofilm. Co-utilization of glucose and xylose by *E. coli* strains is of utmost importance during the production of biofuels, since the fermented plant material contains both, cellulose (polymer of glucose) and hemicellulose (polymer of glucose and xylose), in addition to lignin. Much research is currently dedicated to the genetic modification of *E. coli* that enables the bacteria to utilize xylose more efficiently (Balderas-Hernandez et al., 2010; Hanly & Henson, 2010). It would be interesting to see whether a mixture of our parent strain and its isogenic *flhD* mutant would be able to co-utilize glucose and xylose, particularly since the mutant produced a moderate amount of biofilm which can also be beneficial to the production of biofuels.

5. Conclusion

In summary, we developed an assay system that quantifies biofilm biomass in the presence of distinct nutrients. The assay enables the user to screen a large number of such nutrients for their effect on biofilm amounts. Examples of metabolic analysis relate back to previous literature, as well as giving raise to new hypotheses. Yielding further evidence for the previous hypothesis that acetate metabolism was important in determining biofilm amounts can serve as a positive control that the assay actually yields data of biological significance. Particularly with respect to life in the intestine and the production of biofuels, the data open new avenues of research by providing testable hypotheses. Overall, there is no limit to extensions of the assay into different bacterial species or serving the development of high-throughput data mining algorithms that will computerize the statistic/metabolic analysis that we started in this study.

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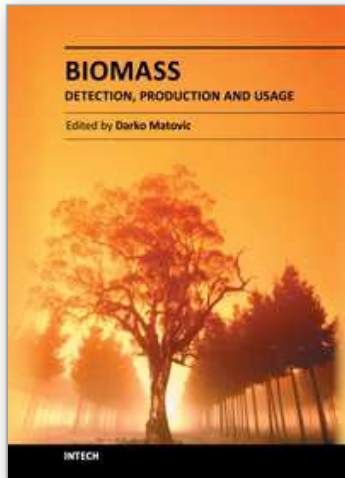
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Biomass has been an intimate companion of humans from the dawn of civilization to the present. Its use as food, energy source, body cover and as construction material established the key areas of biomass usage that extend to this day. Given the complexities of biomass as a source of multiple end products, this volume sheds new light to the whole spectrum of biomass related topics by highlighting the new and reviewing the existing methods of its detection, production and usage. We hope that the readers will find valuable information and exciting new material in its chapters.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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