Effect of Reactor Turbulence on the Binding-Protein-Mediated Aspartate Transport System in Thin Wastewater Biofilms

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This research documents an effect of reactor turbulence on the ability of gram-negative wastewater biofilm bacteria to actively transport l-aspartate via a binding-protein-mediated transport system. Biofilms which were not preadapted to turbulence and which possessed two separate and distinct aspartate transport systems (systems 1 and 2) were subjected to a turbulent flow condition in a hydrodynamically defined closed-loop reactor system. A shear stress treatment of 3.1 N · m⁻² for 10 min at a turbulent Reynolds number (Re) of 11,297 inactivated the low-affinity, high-capacity binding-protein-mediated transport system (system 1) and resolved the high-affinity, low-capacity membrane-bound proton symport system (system 1). The Km and Vmax values for the resolved system were statistically similar to Km and Vmax values for system 1 when system 2 was inactivated either by osmotic shock or arsenate, two treatments which are known to inactivate binding-protein-mediated transport systems. We hypothesize that shear stress disrupts system 2 by deforming the outer membranes of the firmly adhered gram-negative bacteria.

Reactor turbulence is an operational parameter that can be used to improve rates of substrate removal by biofilms in such fixed-film wastewater treatment systems as rotating biological contactors, expanded beds, and fluidized beds. By increasing fluid velocity, and hence reactor turbulence, resistances to substrate diffusion in the boundary layer are minimized, and the intrinsic kinetics of substrate incorporation may be observed. Increased fluid velocities have been shown to improve the rates of substrate incorporation or removal by wastewater biofilms (6, 19, 20, 22, 23, 36, 37). There are, however, detrimental effects associated with increased levels of reactor turbulence. Shear stresses can (i) detach biofilms (4, 5, 32, 36, 37), (ii) affect biofilm composition (25), (iii) compress the biofilm (7, 29), and (iv) detach adsorbed (30) and adhered (13) bacterial cells. There has been no research to date on detrimental effects of shear stress on the cellular physiology of biofilm bacteria. In an accompanying study (14), we observed that a Lineweaver-Burk analysis of initial velocities of aspartate incorporation by thin wastewater biofilms revealed a curvilinear and concave-down plot. This curvilinearity was due to incorporation by two separate and distinct transport systems (systems 1 and 2) rather than to mass transfer limitations or negative cooperation at one transport site. System 1 was resolved by inactivation of system 2 with either osmotic shock or arsenate. System 2 was resolved by inactivation of system 1 with dinitrophenol. System 1 was described as a high-affinity, low-capacity proton symport, and system 2 was described as a low-affinity, high-capacity binding-protein-mediated transporter. System 1 predominated when substrate concentrations were low (less than 40 μM), whereas system 2 predominated when concentrations were high (greater than 80 μM). System 2 was also less specific for aspartate than system 1 in the presence of analogs. We observed that the binding-protein-mediated transporter was probably important for transporting substrate as a carbon source because of its greater capacity and lower specificities. Periplasmic binding proteins are susceptible to inactivation by treatments which increase the permeability of the outer membrane of gram-negative bacteria (12, 17, 28, 33, 38). In previous work (T. T. Eighmy, unpublished data), we observed that increased fluid velocities in a closed-loop reactor system (CLRS) appeared to inactivate system 2, and we suspected that shear stresses associated with turbulent flow were responsible by increasing the permeability of the outer membranes of the firmly adhered gram-negative bacteria. The purpose of this research was to test that hypothesis by subjecting thin biofilms to a defined shear stress and then examining the kinetics of aspartate incorporation under a static condition.

We report a more subtle detrimental effect of shear stress on wastewater biofilms. A shear stress pretreatment of 3.1 N · m⁻² for 10 min at a Reynolds number (Re) of 11,297 was sufficient to inactivate the binding-protein-mediated aspartate transport system (system 2) and resolve the membrane-bound aspartate proton symport (system 1).

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MATERIALS AND METHODS

Biofilm sampling device. We used tubular sampling devices which were suitable for submersión in a wastewater, bacterial colonization, and use in the CLRS. The sampling devices are described elsewhere (14, 15). The only modification to their construction included the use of silicone sealant to seal the threads of the nylon screw so that the seal was watertight. The process used to establish the biofilms in the sampling devices is given elsewhere (14, 15). Biofilms were established at very low wastewater flow velocities. Sampling devices with biofilms of known age (either 16 or 18 days old) were returned to the laboratory and maintained in dilute (10%) primary effluent before use. Loosely bound bacteria were removed from the tubes by gentle rinsing so that only firmly adhering bacteria remained on the stubs. Light microscopic techniques were used to examine the composition of the biofilms before and after a shear stress treatment and to estimate biofilm thicknesses (15).

CLRS. We used the CLRS to subject the adhering biofilms to a shear stress treatment under defined fluid flow condi-
tions. The CLRS was similar in design to those reported elsewhere for studying biofilms under defined flow conditions (4, 5, 9, 25, 26, 29). The CLRS (Fig. 1) consisted of the following: the colonized tube with polyvinyl chloride collars, flexible polyvinyl chloride tubing with access ports, a variable-speed centrifugal pump with a voltage regulator, and a stroboscope. The desired fluid velocity was maintained in the closed loop by adjusting the voltage to the pump so that the pump fan was visually motionless in the beam of the stroboscope set at a predetermined flash rate.

**Fluid hydrodynamics.** Fluid flow through a pipe is well documented (24, 34); consequently, a number of studies have used tubular reactors to study the effects of fluid flow on biofilms (4, 5, 9, 25, 26, 29). We had to determine a number of hydrodynamic parameters associated with a specific fluid velocity and flow condition within the sampling tube to determine the average shear stress. In previous work (T. T. Eighmy, unpublished data), we suspected that a shear stress associated with an Re of 11,297 inactivated the binding-protein-mediated transport system, so we used that highly turbulent fluid flow condition as the shear stress treatment. The definitions and equations describing the Reynolds number (Re, a dimensionless parameter), the mean fluid velocity (\( u \), centimeters per second), the pressure drop (\( \Delta P \), grams per centimeter per square second), the friction factor (\( f \), a dimensionless parameter), the viscous or laminar sublayer thickness (\( \delta \), in centimeters), and the average shear stress (\( \tau_0 \), newtons per square meter) are given elsewhere (6, 25). The mean fluid velocity and the pressure drop were calculated from 20 cumulative flow determinations (\( u \)) at the appropriate voltage setting and from 20 measurements of column height differences (centimeters of water) at that flow in a piezometer interfaced between two pressure ports which were 48 cm apart on a sampling tube. We determined these parameters for the highly turbulent flow condition (Re of 11,297) with both a colonized tube with a 16-day-old biofilm ca. 40 \( \mu \)m thick and an uncolonized (clean) tube.

**Shear stress treatment.** A colonized tube was placed in the CLRS (Fig. 1), and the CLRS was filled with 20°C buffered tap water (BT). The BT solution (pH 6.8) contained 5 mM KH₂PO₄ and 5 mM KH₂PO₄. BT was added to the CLRS so that all air bubbles could be collected and removed via an access port. The pump was operated for 10 min at the predetermined voltage setting to produce the required shear stress. After the treatment, the tube was removed from the CLRS, drained, and placed in 20°C BT before its use in the transport assays.

**Transport assay.** The procedures used for the aspartate transport assays were described elsewhere (14). Briefly, four shear-stress-treated stubs were incubated for 5 min at an appropriate aspartate concentration in 10 ml of an incubation solution containing the appropriate concentration of soluble L-aspartate (0.5 ml) and ca. 3.0 \( \times \) 10⁻² \( \mu \)Ci of the tracer (L-[U-¹⁴C]aspartate) per ml in BT. Untreated controls were run for comparative purposes. Incubations were terminated with 1% buffered Formalin followed by three rinses of BT. Individual stubs were solubilized with a 1:1 Protosol (New England Nuclear Corp., Boston, Mass.)-ethanol mixture and counted with a Beckman 7000 liquid scintillation counter. Disintegrations per minute for each stub were used to calculate the incorporation velocities as a function of biofilm total organic carbon (TOC), as described elsewhere (14). Velocities of incorporation were reported as nanomoles per hour per milligram of TOC. The kinetics of incorporation (\( k \) and \( V_{\text{max}} \)) were determined from a Lineweaver-Burk transformation of the data by a least-squares regression analysis to produce a line of best fit. Both Eadie-Hofstee and Hanes transformations were used for comparison.

**TOC determinations.** The TOC of the adhering biofilm was assayed as described elsewhere (14) by the ampoule method outlined for the Oceanography International model 526 TOC analyzer (Oceanography International Corp., College Station, Tex.). Previous work (T. T. Eighmy, unpublished data) indicated that there were no discernible changes in biofilm TOC between controls and biofilms subject to turbulent shear as long as the biofilms are initially rinsed to remove loosely bound bacteria.

**Chemicals.** L-[U-¹⁴C]aspartate (specific activity, > 200 mCi · mmol⁻¹) and Protosol were purchased from New England Nuclear Corp., Boston, Mass. All other chemicals were reagent grade and were purchased from Sigma Chemical Co., St. Louis, Mo.

**RESULTS**

**Biofilm characteristics and TOC.** The biofilms consisted of a diverse group of firmly adhering gram-negative bacteria as shown elsewhere (14, 15). An extracellular slime mediated adhesion of the rods and filaments to the stub. Both 16- and 18-day-old biofilms were used in this study, which corresponded to 0.22 and 0.23 mg of TOC · cm⁻² of biofilm, respectively (14). These biofilms were ca. 40 to 50 \( \mu \)m thick, as determined by the optical focusing technique (15). There were no apparent changes in biofilm composition or cell density after shear stress treatments. Filaments were still an integral part of the biofilm, and filaments are the morphological types most affected by fluid velocity (25).

**Aspartate incorporation by untreated biofilms.** The aspartate incorporation velocities by untreated biofilms are shown in Fig. 2. The inset graph is the actual plot of the velocity of incorporation versus aspartate concentration. The Lineweaver-Burk plot of that data is curvilinear, which indicated that both transport systems (systems 1 and 2) were present in the biofilm (14). The Eadie-Hofstee and Hanes transformations of the data resulted in curvilinear plots with good correlation as well.

**Fluid hydrodynamics.** Results of the hydrodynamic analyses of the CLRS with a colonized tube are as follows (the observed values were identical to tests performed with
UNCOLONIZED TUBES). UNDER TURBULENT FLUID CONDITIONS (BASED ON COMPLETE TRANSITION TO TURBULENT FLOW AT RE = 2,300 [34]) AT THE RE OF 11,297, THE MEAN VELOCITY THROUGH THE SAMPLING TUBE WAS 0.89 M · S⁻¹, AND THE ASSOCIATED FRICTION FACTOR OF 3.10 × 10⁻² AGREED WITH VALUES REPORTED FOR SMOOTH PIPES. THE VISCOUS SUBLAYER THICKNESS, δ, WAS 90 MUM WHICH WAS MUCH GREATER THAN THE HYDRATED BIOFILM THICKNESSES (40 TO 50 MUM) OF THE 16- AND 18-DAY-OLD BIOFILMS. MCCOY ET AL. (25) AND CHARACKLIS (7) NOTE THAT SIGNIFICANT INCREASES IN THE PRESSURE DROP OCCUR WHEN BIOFILM THICKNESS EXTENDS OR FILAMENTOUS CELLS GROW BEYOND THE VISCOS SUBLAYER. WHEREAS FILAMENTS WERE A COMPONENT OF THE BIOFILMS WE WORKED WITH, THEIR PRESENCE DID NOT ALTER THE HYDRO_DYNAMIC PERFORMANCE OF THE CLRS, AS PRESSURE DROPS WERE IDENTICAL BETWEEN COLONIZED AND UNCOLONIZED TUBES. (ΔP FOR OUR ANALYSIS WAS 4,695 G · CM⁻¹ · S⁻².) THE AVERAGE SHEAR STRESS, τ₀, ASSOCIATED WITH AN RE OF 11,297 WAS 3.1 N · M⁻², AND COLUMN HEIGHT (IN CENTIMETERS OF WATER) WAS 4.80.

ASPARTATE INCORPORATION BY SHEAR-STRESSED BIOFILMS. THE ASPARTATE INCORPORATION VELOCITIES BY SHEAR-STRESS-PRETREATED BIOFILMS IS SHOWN IN FIG. 3. PRETREATMENT WITH A SHEAR STRESS OF 3.1 N · M⁻² FOR 10 MIN WAS SUFFICIENT TO INACTIVATE THE BINDING-PROTEIN-MEDIATED SYSTEM AND RESOLVE THE MEMBRANEBOUND SYSTEM. THE KINETIC CONSTANTS FOR ASPARATE TRANSPORT BY THE RESOLVED SYSTEM (SYSTEM 1) ARE SHOWN IN TABLE 1 AND ARE COMPARED WITH OTHER TREATMENTS (ARSENATE OR OSMOTIC SHOCK) WHICH INACTIVATE BINDING-PROTEIN-MEDIATED TRANSPORT SYSTEMS AND WHICH ALSO INACTIVATED SYSTEM 2 AND RESOLVED SYSTEM 1 IN AN ACCOMPANYING STUDY (14). THE EADIE-HOFSTEER AND HANES TRANSFORMATIONS OF THE DATA FOR SYSTEM 1 BASED ON SHEAR STRESS INACTIVATION OF SYSTEM 2 PRODUCED KINETIC CONSTANTS SIMILAR TO THOSE OF THE LINeweaver-Burk TRANSFORMATION (TABLE 1).

DISCUSSION

This study shows that reactor turbulence is capable of inactivating the binding-protein-mediated aspartate transport system in wastewater biofilms. This work is significant in demonstrating that shear stresses associated with reactor turbulence can produce a subtle detrimental effect on the physiology of gram-negative bacteria which constitute an adhering wastewater biofilm.

We believe that shear stress was the mechanism for inactivating the binding-protein-mediated aspartate transport system (system 2) for the following reasons. (i) Biofilms subjected to shear stress before their incubations in the aspartate transport assays did not exhibit curvilinearity in Lineweaver-Burk transformed data, whereas unstressed controls possessing both systems did, indicating the loss of one transport system from the shear stress-treated biofilms. (ii) The kinetics of aspartate incorporation by the resolved system (system 1) were statistically similar to the kinetic constants derived for system 1 after metabolic (arsenate) or osmotic shock treatments which were used to inactivate the binding-protein-mediated system (14). A randomized complete block design analysis (27) of ranked Kᵣ or V_max values revealed that there were no significant differences at the 0.01

FIG. 2. Lineweaver-Burk plot of aspartate incorporation by untreated biofilms. The mean values (C) were averaged from individual incorporation velocities by four stubs at each substrate concentration. The average percent standard deviation about the means was 27.5. The inset is a plot of the velocity versus substrate concentration of the data represented in the Lineweaver-Burk plot.

FIG. 3. Lineweaver-Burk plot of aspartate incorporation by system 1 as revealed by shear stress inactivation of system 2. The mean values (C) were averaged from individual incorporation velocities by eight stubs at each substrate concentration. The average percent standard deviation about the means was 25.8. The least-squares regression for the data produced the line of best fit (—) for the data. See footnotes to Table 1 for explanation of Kᵣ, V_max, and r.

TABLE 1. Apparent kinetic constants of system 1 based on a variety of inactivation treatments of system 2

<table>
<thead>
<tr>
<th>Systemبك (TREATMENT)</th>
<th>Transformation</th>
<th>Kᵣ (μM)</th>
<th>V_max (nmol h⁻¹ mg of TOC⁻¹)</th>
<th>r²</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (AsO₄²⁻)</td>
<td>LB</td>
<td>4.3</td>
<td>5.9</td>
<td>0.97</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>5.1</td>
<td>6.4</td>
<td></td>
<td>VG</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>14.1</td>
<td>8.7</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>1 (osmotic shock)</td>
<td>LB</td>
<td>4.6</td>
<td>5.9</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>4.1</td>
<td>5.9</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>3.9</td>
<td>6.7</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>1 (shear stress)</td>
<td>LB</td>
<td>3.6</td>
<td>6.7</td>
<td>0.96</td>
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</tr>
<tr>
<td></td>
<td>EH</td>
<td>3.9</td>
<td>7.2</td>
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<td>VG</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>8.9</td>
<td>8.6</td>
<td>0.99</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Based on linear regressions or subjective line of best fit on mean incorporation velocities from data presented previously (14) and in the legend to Fig. 3.
* Kᵣ, Michaelis-Menten constant; V_max, maximum velocity of the system.
* Refers to resolved system.
* Refers to method used to resolve the system.
* Methods of linearization of the data abbreviated as follows: LB, Lineweaver-Burk; EH, Eadie-Hofsteer; and H, Hanes transformations.
* Correlation coefficient for linear regressions; with EH plot, the goodness of fit is subjectively described by the following abbreviations: VG, very good; G, good.
level between the types of treatments or linearizations for the data presented in Table 1.

Periplasmic binding proteins are soluble, single polypeptide chains with a molecular weight between 26,000 and 41,000 that facilitate active transport for a variety of substrates (12, 35, 38). They are inactivated or removed from the periplasm by treatments which disrupt or increase the permeability of the lipopolysaccharide-containing outer membrane (17, 28, 33, 38). Furthermore, the reconstitution of binding proteins into the periplasm requires an increase in the permeability of the outer membrane to allow the import of the proteins (1–3), and lipopolysaccharide-deficient mutants of Salmonella typhimurium do not retain periplasmic enzymes because of the increased permeability of the lipopolysaccharide-deficient outer membrane (10). We feel that fluid downsweeps (8), which are associated with turbulent flow in the CLRS, applied shear forces to the adhering cells and their extracellular glyocalcyes and caused the disruption of the binding-protein-mediated transport system by deforming the plastic outer membrane (11).

There is evidence which supports our hypothesis that a shear stress of 3.1 N m⁻² is dynamic enough to buffet and deform the biofilm but not to detach the firmly adhering bacteria. Shear stress affects the rate of biofilm growth (25, 26) and detachment (4, 5, 32). Fluid shear can also affect the microbial composition of the biofilm (25). Shear stress increases from 2 to 15 N m⁻² cause four- to fivefold increases in biofilm densities by squeezing pore water from the biofilm (7, 29). Shear stresses of greater than 6 N m⁻² drastically reduce the number of Pseudomonas fluorescens cells attempting to adhere to a substratum, and shear stresses of greater than 10 N m⁻² greatly increase the detachment rate of firmly adhered P. fluorescens (13). Soil isolates also show a critical shear stress (between 2.6 and 5.4 N m⁻²) below which significant deposition and adhesion occurs, depending on the surface properties of the substrata (16). Adsorbed Bacillus spp., Escherichia coli, and S. typhimurium are also detached by shear stresses ranging from 1.1 to 44.0 N m⁻² (30). Very low shear stresses (0.092 and 0.8 N m⁻²) can affect the residence time of Bacillus cereus cells which are deposited, adsorbed, and detached from a substratum under laminar flow conditions (31).

In fixed-film wastewater treatment processes, increased fluid velocities are employed to increase reactor turbulence so that substrate removal rates can be improved (6, 19, 20, 22, 23, 36, 37). The shear forces associated with these velocities, which can easily exceed the value of 3.1 N m⁻² used in this work, can inactivate the binding-protein-mediated transport systems in the biofilm bacteria. The implications of this are that biofilm bacteria may lose the ability to transport a substrate as a carbon source, thus impairing cell growth and maintenance functions. Although the susceptibility of these transport systems in shear stress-adapted biofilms needs to be determined, the implications of this research still apply to stream epithelial biofilms (18), fouling biofilms in heat exchangers and water mains (7, 8), and biofilms in immobilized cell reactors (21). In conclusion, our results suggest that there are detrimental effects on biofilm physiology attributed to turbulent shear stresses in fixed-film systems.

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LITERATURE CITED


