**Pseudomonas aeruginosa** inactivation mechanism is affected by capsular extracellular polymeric substances reactivity with chlorine and monochloramine

Zheng Xue¹, Christopher M. Hessler², Warunya Panmanee³, Daniel J. Hassett³ & Youngwoo Seo¹,²

¹Department of Civil Engineering, University of Toledo, Toledo, OH, USA; ²Department of Chemical and Environmental Engineering, University of Toledo, Toledo, OH, USA; and ³Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH, USA

**Correspondence:** Youngwoo Seo, Department of Civil Engineering, University of Toledo, 2801 W. Bancroft, St Toledo, OH 43606, USA. Tel.: +1 419 530 8131; fax: +1 419 530 8116; e-mail: youngwoo.seo@utoledo.edu

Received 1 March 2012; revised 12 July 2012; accepted 12 July 2012.
Final version published online 8 August 2012.
DOI: 10.1111/j.1574-6941.2012.01453.x

**Editor:** Cindy Nakatsu

**Keywords**
extracellular polymeric substances; inactivation kinetic; EPS reactivity; chlorine; monochloramine.

**Abstract**
The reactivity of capsular extracellular polymeric substances (EPS) to chlorine and monochloramine was assessed and compared in this study. The impact of capsular EPS on Gram-negative bacteria *Pseudomonas aeruginosa* inactivation mechanisms was investigated both qualitatively and quantitatively using a combination of batch experiments, viability tests with LIVE/DEAD staining, and Fourier transform infrared spectroscopy (FTIR). Both wild-type and isogenic mutant strains with different alginate EPS production capabilities were used to evaluate their susceptibility to chlorine and monochloramine. The *macA22* mutant strain, which overproduces the EPS composed largely of acidic polysaccharide alginate, exhibited high resistance and prolonged inactivation time to both chlorine and monochloramine relative to PAO1 (wild-type) and *algT(U)* mutant strains (alginate EPS deficient). Multiple analyses were combined to better understand the mechanistic role of EPS against chlorine-based disinfectants. The extracted EPS exhibited high reactivity with chlorine and very low reactivity with monochloramine, suggesting different mechanism of protection against disinfectants. Moreover, capsular EPS on cell membrane appeared to reduce membrane permeabilization by disinfectants as suggested by deformation of key functional groups in EPS and cell membrane (the C–O–C stretching of carbohydrate and the C=O stretching of ester group). The combined results supported that capsular EPS, acting either as a disinfectant consumer (for chlorine inactivation) or limiting access to reactive sites on cell membrane (for monochloramine inactivation), provide a protective role for bacterial cells against regulatory residual disinfectants by reducing membrane permeabilization.

**Introduction**
The protection of public water supplies against waterborne diseases greatly relies upon the use of disinfectants. However, reports from many water utilities in the United States have shown that pathogens can survive in water distribution systems, despite the presence of residual disinfectants (Jjemba et al., 2010).

A growing number of studies have discussed the influence of extracellular polymeric substances (EPS) produced by microorganisms on bacterial regrowth and biofilm formation in water systems (Momba et al., 2000). The presence of EPS has been known to facilitate bacterial immobilization, proliferation, reattachment, and regrowth. EPS have significant impact on bacterial surface characteristics, which may alter biochemical properties of bacterial cells (Madigan & Martinko, 2006; Liu et al., 2007a).

Additionally, it is well accepted that EPS provide protection against inhospitable environments, such as antibiotics, desiccation, and heavy metals. (Wingender et al., 1999). Several researchers have reported that the presence of EPS enhanced bacterial viability against bactericidal activities (Liu et al., 2007b; Mitchell et al., 2008).
It was also found that extracellular alginate has an intrinsic protective effect on suspended *Pseudomonas aeruginosa* cells against antibiotics by a non-charge-related binding mechanism (Hodges & Gordon, 1991). In addition to physical attributes that aid in resistance of microorganisms to free chlorine, many bacterial species defend against oxidative stresses by adopting resistance mechanisms that increase EPS production (Helbling & VanBriesen, 2007). It was previously hypothesized that extracellular material may sacrificially react with free chlorine and reduce the concentration of disinfectant at the cell membrane. Furthermore, a debate persists whether the microbial inactivation by free chlorine involves changes in membrane permeability or extensive oxidation of the microbial cell membrane, which results in leakage of intracellular material and cell death (Hurst et al., 1991; Virto et al., 2005). However, the reactivity of extracellular material on the cell membrane with disinfectants was not considered in the debated inactivation mechanisms.

Despite growing acceptance of protective role of EPS against the act of antimicrobial agents, previous studies predominantly focused on monitoring the bacterial survival rate and disinfectant consumption, without consideration of the physiochemical properties of the microorganism. Specifically, the chemical composition or the functional groups of bacterial EPS involved in the reaction with disinfectants were not well characterized (Chhabra et al., 2005), although most bacterial strains produce EPS even as suspended cultures (Beech et al., 1999; Wingender et al., 1999; Laspidou & Rittmann, 2002). Currently, no systematic study has been conducted to illustrate the reactivity of EPS with disinfectants or the role of EPS in microbial inactivation (Liu et al., 2007b; Mitchell et al., 2008). Furthermore, there has been increasing interest in alternative disinfectants to free chlorine, such as chloramines, to reduce disinfectant byproduct formation. The role of capsular EPS in inactivation mechanisms of chloramines was not robustly evaluated, specifically considering the reactivity between EPS and chloramines (Gagnon et al., 2004).

In this study, we sought to assess and compare the reactivity of capsular EPS to chlorine and monochloramine using both qualitative and quantitative analysis. Three *P. aeruginosa* strains, both wild-type and isogenic mutants with varied alginate EPS production, were evaluated to assess the role of EPS on bacterial inactivation. Simulating minimal regulatory disinfectant residuals in water distribution systems, inactivation kinetics, and disinfectant consumption rates of the EPS-producing variants were studied at low disinfectant concentrations. Examining both whole cells and extracted EPS in batch experiments, changes in the EPS quantity and composition as a response to disinfectant exposure were monitored.

### Materials and methods

#### Bacterial culture

*Pseudomonas aeruginosa* is an opportunistic human pathogen, and cases of contamination in water distribution systems, as well as in food-processing equipment, were attributed to this particular organism (Geldreich, 1989; Hardalo & Edberg, 1997; DeQueiroz & Day, 2007). All isogenic mutant strains were constructed in wild-type *P. aeruginosa* strain PAO1, whose primary EPS component is the acidic polysaccharide alginate. Two mutants, mucA22 (overproduction of alginate EPS) and algT(U) (minimal or no alginate EPS production), were constructed as described elsewhere (Schweizer, 1993; Schweizer & Hoang, 1995). All strains were grown in one-tenth strength LB broth (2.5 g L$^{-1}$; Difco Laboratory, Detroit, MI) at 37 °C until the late-exponential phase and then harvested by centrifugation at 2000 g for 15 min (model 5804R centrifuge; Eppendorf, Hamburg, Germany), allowing for minimal removal of capsular alginate EPS (Eboigbodin & Biggs, 2008), washed twice, and resuspended in chlorine demand-free (CDF) buffer (0.54 g Na$_2$HPO$_4$ and 0.88 g KH$_2$PO$_4$ L$^{-1}$, pH 6.98; Thurston-Enriquez et al., 2003) as bacterial suspensions (c. $10^8$ cells mL$^{-1}$). The cell density selected for batch disinfection experiments is consistent with those employed in other studies (Ridgway & Olson, 1982; Luh & Marinas, 2007; Vicuna-Reyes et al., 2008). Additionally, the cell density selected for batch disinfection experiments also reflects biofilm cell densities found in distribution systems (Lechevallier et al., 1988; Carter et al., 2000).

#### Preparation of disinfectant solutions

All disinfection experiments were conducted with CDF buffer. Chlorine solution (0.5 mg L$^{-1}$) was prepared with Clorox bleach (The Clorox Co., Oakland, CA). Monochloramine solution (2 mg L$^{-1}$) was prepared by combining solutions of sodium hypochlorite (5.65–6%, pH 8.3) and ammonium chloride (0.2 mM, pH 8.3) in a 4 : 1 chlorine-to-ammonia-nitrogen mass ratio, where monochloramine is the dominant species at this pH. The disinfectant solutions were prepared immediately preceding the inactivation experiments. The disinfectant concentrations were selected based on residual disinfectant concentration typically used in water distribution systems (USEPA, 1999). Both the initial doses and residual concentrations were measured as free chlorine by N,N-diethly-p-phenylenediamine (DPD) method for
chlorine and indophenol method for monochloramine using a DR/2700 spectrophotometer (HACH Company, Loveland, CO; Qiang et al., 2006).

**Batch disinfection experiments**

All batch experiments were performed in 250-mL amber glass bottles (Fisher Scientific) at room temperature (22 °C ± 2). Amber glass bottles were used as parallel reactors, where duplicate sample bottles containing culture suspension and disinfectant were compared with a control bottle. Inactivation tests with disinfectants were conducted simultaneously measuring disinfectant decay and bacterial survival. After quenching residual disinfectant with CDF buffer solution containing Na₂S₂O₃ (1 mM), enumerations of viable microbial cells were performed using the plate count method with R2A agar plates (Difco Laboratories, Detroit, MI). Batch experiments were repeated more than three times for each strain with each disinfectant, and each sample was plated in duplicate.

**Modeling with Geeraerd and Van Impe inactivation model-fitting tool**

The Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT, Version 1.5; KULeuven, Belgium) was used to interpret non-log linear microbial survival models on inactivation data (Geeraerd et al., 2005). The best-fit model was chosen by comparing the values of the root mean square of errors as described previously (Berney et al., 2006). The model applied was a log-linear model with shoulder and tailing for monochloramine and with tailing for chlorine disinfection (Eqn. 1; Geeraerd et al., 2005). Based on the model-fitted data, Ct values (concentration of disinfectant × time) for 2-log inactivation (99% of the population is inactivated) were calculated.

\[
N(t) = \left( N(0) - N_{\text{res}} \right) \frac{\exp(-K_{\text{max}}t) \cdot \exp(K_{\text{max}}S_1)}{\left[ 1 + \exp(K_{\text{max}}S_1) - 1 \right] \cdot \exp(-K_{\text{max}}t)} + N_{\text{res}} \tag{1}
\]

Where, \(K_{\text{max}}\) (1/time unit) is the specific inactivation rate. \(S_1\) (time unit) is the lag time representing the shoulder (\(S_1 = 0\) for chlorine inactivation). \(N_{\text{res}}\) is the number of more resistant population representing the tailing.

**Bacteria cell staining**

The LIVE/DEAD Bacterial Viability kit (BacLight; InVitrogen by Life Technologies, Carlsbad, CA) was applied to enumerate both viable and total counts of bacteria investigating disinfectant efficacy. In the LIVE/DEAD stain kit, SYTO 9 penetrates all bacterial membranes and stains the cells green, while propidium iodide (PI) only penetrates cells with damaged membranes. The combination of the two stains stoichiometrically produces red fluorescing cells. When the BacLight viability stain is compared with traditional colony-forming unit counts, potentially viable but nonculturable (VBNC) cells can be identified as live, as long as they have intact cell membrane. Combining Live/Dead staining and plate count methods provided information about the enumeration of culturable and nonculturable bacteria, but also membrane-compromised injured cells. This reflected different levels of damage to the cell membrane because of chemical disinfection. Reports from previous studies have shown that the staining of bacterial cells with SYTO 9 and PI did not always produce distinct ‘live’ and ‘dead’ populations (Stocks, 2004; Berney et al., 2007). The appearance of yellow- or orange-stained cells observed in this study indicated an intermediate state of membrane-compromised cells and was similarly included with the red cell population. Cells that can only be observed in the green fluorescence channel were defined as viable.

Bacterial suspensions were exposed to disinfectants and quenched with sodium thiosulfate (1 mM) when c. 90% inactivation was achieved, as determined from GInaFiT curve-fitting results. The 90% inactivation level was chosen for Live/Dead staining experiments, because it was difficult to properly differentiate viability of the three strains at 99% inactivation phase. Bacterial cells, particularly those that were alive, were more clearly stained at 90% inactivation. When 99% inactivation was examined, bacteria tended to be either red stained or orange and yellow stained, because of complete or partial cell membrane injury. Stained solution was filtered through black polycarbonate membrane filters (0.45 μm pore size; Millipore) for fluorescence microscopic imaging. Fluorescent images were observed at 480/500 nm for SYTO 9 and 488/617 nm for PI, respectively. The ratios of viable cell counts (green) to total cell counts were derived from at least 20 images for each sample, and experiments were repeated at least twice for each bacterial strain. A fluorescent microscope (IX51, Olympus) equipped with a 100× oil immersion objective was used for image acquisition. IMAGEJ (http://rsb.info.nih.gov/ij/) and CELLANALYST (Assay-Soft Inc., Fountain Valley, CA) were used for image processing and cell counting.

**EPS extraction and characterization**

EPS extraction of each strain was performed in triplicate on bacterial suspensions prepared from an initial 200-mL bacterial culture harvested at the late-exponential phase (c. 16 h). The modified EDTA extraction method combined with high-speed centrifugation was employed.
as described elsewhere (Brown & Lester, 1980). The supernatant from extraction was collected and quantified for total protein and polysaccharide content in EPS.

Protein concentrations were determined using the modified Lowry Protein Assay kit using Coomassie Blue (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as standard. Total polysaccharide concentration was measured using the phenol–sulfuric acid method using glucose as standard (Brown & Lester, 1980). Alginate content of the extracted EPS was specifically quantified using the H$_2$SO$_4$–borate method with sodium alginate as a standard (Knutson & Jeanes, 1968).

**Disinfectant consumption by extracted EPS**

The extracted EPS were diluted to a series of concentrations ranging from 0 to 200 µg mL$^{-1}$ based on alginate concentration and reacted with chlorine and monochloramine in 1 : 1 volume ratio, respectively. In addition, the same concentration range of BSA was prepared and interacted with monochloramine as described previously for comparison. The initial disinfectant concentration and total exposure time were identical to batch inactivation experiments. At each sampling point, disinfectant concentrations were measured to determine the reaction kinetics using colorimetric methods described earlier. A two-phase decay model adapted from the EPA 1998 model (Equation 2) was used to evaluate reactivity of alginate and tested disinfectants (Sohn et al., 2004). In this model, the rate constants $k_1$ and $k_2$ were assigned for decay rates of the two phases during disinfectant decay, where $k_1$ dominates the fast decay phase and $k_2$ dominates the slow decay phase.

$$C = C_0 \times [A \times e^{-k_1t} + (1 - A) \times e^{-k_2t}]$$

**FTIR spectroscopy**

Previous studies have shown that IR spectroscopy could be used in microbial disinfection research (Al-Qadiri et al., 2008). In this study, FTIR was employed to probe both the chemical composition of bacterial cells and extracted EPS, but also to monitor their alterations (infrared absorbance intensity) induced by the two disinfectants. Batch experiment procedures were followed to disinfect and quench samples. Bacterial cells were harvested by filtering 15 mL of each suspension through a 0.2-µm aluminum oxide membrane filter (Anodisc; Whatman Inc., NJ), which has no IR absorbance in the range of 4000–600 cm$^{-1}$. In EPS reactivity experiments, EPS samples were prepared with an initial concentration of 50 µg alginate mL$^{-1}$ and reacted with disinfectants following batch experiment exposure time corresponding to 2-log reduction. The EPS samples were evenly spread on stainless steel, which contributes no IR spectra, and air-dried overnight. The dried samples were analyzed using FTIR in microattenuated total reflection (ATR) mode. The distinctive functional groups of capsular EPS and inner cell substances could be identified by subtracting the extracted EPS spectra from the bacterial cell spectra.

A FTS-4000 Varian Excalibur Series FTIR spectrometer (Varian, Palo Alto, CA) was used for collecting the infrared spectra. Spectra from 4000 to 400 cm$^{-1}$ were collected with a resolution of 2 cm$^{-1}$, and the ordinate was expressed as absorbance. Each spectrum was an average of 256 scans; over six spectrums were collected for each sample. For further data analysis, collected spectra were smoothed with a Gaussian function to eliminate system noise, followed by a second-derivative transformation to amplify the compositional and structural changes in bacterial cellular constituents (Holt et al., 1995).

**Statistical analysis**

Data were presented as mean ± standard deviation. Differences were analyzed with unpaired t-test or one-way ANOVA test at 0.05 significance level using SIGMAPLOT (Jandel Scientific, Sausalito, CA).

**Results**

**Culture and EPS properties**

The analysis of the two major components of extracted EPS, protein and polysaccharide, provided evidence of varied EPS production capacities of the three strains and confirmed with previous report (Tielen et al., 2010). The total carbohydrate content was 6.71 × 10$^{-11}$, 1.62 × 10$^{-8}$, and 2.46 × 10$^{-8}$ µg per cell for algT(U), PAO1, and mucA22 strain, respectively. The protein content was in a lower magnitude compared with the carbohydrate content, which was 2.18 × 10$^{-12}$, 4.38 × 10$^{-9}$, and 6.62 × 10$^{-9}$ µg per cell for the three strains, respectively. The SEM imaging of the tested strains clearly demonstrated a difference in surface morphology, which is attributed to capsular EPS (Supporting information, Fig. S1).

**Inactivation kinetics**

Batch experimental results were interpreted as the fraction of culturable bacteria [Log ($N/N_0$)] versus Ct values, which were used to assess the susceptibility of cells to disinfectants when a 2-log (99%) inactivation occurred.
Repeated tests showed that all three strains were immediately sensitive to chlorine exposure but lagged in response to monochloramine. The inactivation of the three P. aeruginosa strains by 0.5 mg L\(^{-1}\) of chlorine was rapid (< 1 s) for 99% reduction (Fig. 1a). However, more than 30 min was required to reach the same level of inactivation for all three strains studied upon reaction with 2 mg L\(^{-1}\) of monochloramine (Fig. 1b).

Batch experiments with the three P. aeruginosa strains revealed inactivation in the presence of disinfectant occurred at a lower rate for the alginate EPS–overproducing strain compared with the wild-type and alginate EPS–underproducing strain, given by average Ct values summarized in Table 1. The 99% inactivation Ct values of the three strains were compared, using wild-type PAO1 strain with normal EPS production as a baseline. The Ct value required for 99% chlorine inactivation in \(\text{algT(U)}\) was 20% lower, and Ct value of \(\text{mucA22}\) was three times higher than that of PAO1. For monochloramine inactivation, the comparative result was 10% lower \(\text{algT(U)}\) and 18% higher \(\text{mucA22}\).

**Model fitting**

Table 2 shows the summary of disinfection kinetic parameters derived from inactivation curve fitting conducted with GINFIT. Monochloramine inactivation tests revealed a lag time at the initial period of inactivation followed by rapid inactivation and then a tail for all strains. The shoulder length (SL) was calculated by multiplying lag time with monochloramine concentration. The \(\text{algT(U)}\) strain showed shortest lag time (13 min) followed by PAO1 (25 min) and \(\text{mucA22}\) (32 min). Inactivation rate could be evaluated using the \(K_{\text{max}}\) value calculated from the model (Eqn. 1). A higher \(K_{\text{max}}\) indicates a more rapid inactivation rate. The model-fitting results showed that the \(\text{algT(U)}\) strain exhibited highest \(K_{\text{max}}\) during inactivation and least survival in tail, followed by PAO1 and \(\text{mucA22}\) strain. In chlorine inactivation experiments, which exhibited rapid inactivation rate followed by a tail with no lag time, \(\text{mucA22}\) was comparatively more resistant, signified by the lowest \(K_{\text{max}}\) [74% lower than \(K_{\text{max}}\) for PAO1]. In comparison, \(\text{algT(U)}\) exhibited the

### Table 1. Ct values by plate counting and viable ratio by fluorescent staining

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Chlorine (Ct (mg min L(^{-1}))(^*))</th>
<th>Viable bacteria ratio (^\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{algT(U)})</td>
<td>0.0073 ± 0.01</td>
<td>68.40 ± 0.27</td>
</tr>
<tr>
<td>PAO1</td>
<td>0.0091 ± 0.01</td>
<td>75.95 ± 0.44</td>
</tr>
<tr>
<td>(\text{mucA22})</td>
<td>0.038 ± 0.00</td>
<td>89.83 ± 0.49</td>
</tr>
</tbody>
</table>

\(^*\)Ct values required for 99% inactivation according to inactivation kinetic model fitting.

\(^\dagger\)Ratio of live cell versus total cell at the 90% inactivation time obtained from plate counting. The results are expressed as mean ± standard deviation of two independent experiments in duplicate (\(N\geq 80\)). All pairwise comparisons among the three tested strains were statistically significant in each group (\(P<0.05\), Tukey test).

### Table 2. Disinfection kinetic parameters from inactivation model fitting

<table>
<thead>
<tr>
<th></th>
<th>(\text{algT(U)})</th>
<th>PAO1</th>
<th>(\text{mucA22})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE*</td>
<td>Mean ± SE*</td>
<td>Mean ± SE*</td>
</tr>
<tr>
<td>Chlorine disinfection</td>
<td>(K_{\text{max}}) (min(^{-1}))</td>
<td>118.80 ± 24.43</td>
<td>107.60 ± 20.68</td>
</tr>
<tr>
<td>Monochloramine disinfection</td>
<td>SL (mg min L(^{-1}))</td>
<td>26.75 ± 6.48</td>
<td>50.22 ± 3.68</td>
</tr>
<tr>
<td></td>
<td>(K_{\text{max}}) (min(^{-1}))</td>
<td>0.25 ± 0.05</td>
<td>0.17 ± 0.02</td>
</tr>
</tbody>
</table>

\(^*\)SE, standard error. The results are expressed as mean ± standard error of three independent experiments in duplicate (\(N\geq 12\)).

\(^\dagger\)SL, shoulder length. Units correspond to Ct value. Division of SL by concentration provides inactivation lag time. Values not followed by a common letter are statistically different from each other (\(P<0.05\), Tukey test).
highest $K_{\text{max}}$ [10% higher than $K_{\text{max}}$ for PAO1], representing the highest inactivation rate and most susceptibility to disinfectants.

**Viability analysis by fluorescent staining**

Bacterial viable ratio was expressed in the form of the mean and standard deviation outlined in Table 1. Based on the one-way ANOVA test, there was a difference in viable ratio among these three strains ($P < 0.05$). In chlorine inactivation, viable ratio of mucA22 strain was 5.3 times higher than that for PAO1, while $algT(U)$ survival was not significant. For monochloramine inactivation, the viable ratio of PAO1 was 39% lower than mucA22 and 11% higher than $algT(U)$ strain.

**Disinfectant consumption**

Figures 2 and 3 illustrate disinfectant consumption rates for the three *P. aeruginosa* strains and extracted EPS. For the whole-cell disinfectant consumption test (Fig. 2), chlorine was rapidly consumed during the initial period of inactivation. The residual concentrations after 99% of inactivation were similar for all three strains. Limited by short inactivation times and high reactivity, significant differences in disinfectant residual among strains were not observed. Monochloramine, known to be a slow-reacting disinfectant, was consumed differently by bacterial cells with varying EPS amount ($P < 0.05$).
algT(U) mutant consumed the greatest amount of monochloramine, while higher levels of residual monochloramine were observed in the mucA22 inactivation tests. For the disinfectant consumption test with extracted alginate-based EPS, rapid reaction with chlorine was evident within short time periods (Fig. 3a). However, EPS reactions with monochloramine were slower, even under an extended exposure time (Fig. 3b). Compared with the extracted EPS, BSA reacted much faster with monochloramine (Fig. 3c), where monochloramine concentration declined quickly within the first 20 min of interaction and then consumption slowed for the remaining exposure.

**FTIR analysis**

Infrared absorbance intensities of both the bacterial cells and the extracted EPS spectra were monitored to evaluate alterations and reaction of capsular EPS (functional group changes) with disinfectants. Figure 4 depicts the representative FTIR spectra (4000–400 cm⁻¹) of the untreated and disinfectant-treated (2-log inactivation) P. aeruginosa bacteria cells (Fig. 4a for chlorine; Fig. S2 for monochloramine) and extracted EPS (Fig. 4b). The spectral bands primarily impacted by disinfectant exposure were located at 3700–2700 and 1800–900 cm⁻¹. Characteristic band assignment for FTIR spectra are based on previous studies (Table S1; Holt et al., 1995; Kansiz et al., 1999).

Figure 4(b) shows functional group contributions of extracted EPS and their response to disinfectant exposure. The polysaccharide alginate has been linked to biofilm bacteria resistance against antimicrobial agents that include many frontline antibiotics (Hentzer et al., 2001). Previous reports indicate that the extracellular alginate is composed of β-1, 4-linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Wingender et al., 1999). The presence of these acids in the extracted EPS can be identified from their characteristic bands: the C–O–C stretching of carbohydrate at 1025 cm⁻¹, the C=O stretching of ester group at 1600 cm⁻¹, the CH₂/CH₃ vibration at 1425 cm⁻¹.

Gaussian smoothed and second-derivative-transformed spectra for chlorine-treated bacterial samples are included in Fig. 5 (monochloramine spectra in Fig. S3). There were clear differences in the spectral intensity variation among three strains in the region between 1500 and 900 cm⁻¹, indicating the extent of O–H and C–H stretch deformation in carbohydrate bands. Overall, the mucA22 mutant showed the largest absorbance reduction after disinfection. There was an obvious peak decrease in the 1460–1200 and 1160–1000 cm⁻¹ range, indicating the associated reduction in alginate and other polysaccharide band intensities. Additionally, there were peak changes associated with proteins in the amide I and amide II regions. The mucA22 mutant strain showed significant decrease in amide band vibration followed by the wild-type strain PAO1 and the algT(U) strain. In general, chlorine was more reactive than monochloramine, thus greater absorbance intensity reductions occurred for all three strains after exposure. The mucA22 mutant had an absorbance intensity reduction up to 60–70% on average, compared with 40–55% for wild-type strain PAO1 and 20–35% for the algT(U) mutant.

**Discussion**

In this study, the EPS-overproducing strain consistently yielded a proportionally greater number of culturable cells and higher viability rate, compared with wild-type and EPS-nonproducing strains against tested disinfectants.
Meanwhile, removal of the EPS greatly increased the susceptibility of the mucA22 strain to both chlorine and monochloramine (Fig. S4).

A unique observation in this study was disinfectant consumption by bacterial cells and EPS components. It has been suggested that chlorine reacts differently than monochloramine as disinfectant (Connell, 1996). Chlorine is a nonselective reactant, rapidly reacting with both organics and inorganics. In comparison, monochloramine is found to be more stable in the water distribution system and provides better control of bacterial regrowth than chlorine. Monochloramine deactivates bacteria by penetration of the cell wall, reacting with amino acids, and disrupting bacterial metabolism (Connell, 1996). Jacangelo et al. (1991) reported that inhibition of typical protein-associated biological activities, for example, bacterial transport, respiration, and substrate dehydrogenation, was observed at monochloramine concentrations normally used for disinfection. Based on the two-phase disinfectant decay model, the rate constant \( k_1 \) dominating the fast decay phase of chlorine–alginate (Fig. 3a) and monochloramine–BSA (protein surrogate; Fig. 3c) was found to be as high as 0.2–1.8 \((\text{min}^{-1})\), whereas the same parameter for natural organic matter reactivity with chlorine was previously reported around 0.02–0.04 \((\text{min}^{-1})\) (Gang et al., 2003).

In monochloramine disinfection, alginate-based EPS, a polysaccharide with low observed monochloramine demand (Fig. 3c), could limit access to reactive sites on the bacterial cell membrane, which is more reactive with monochloramine than the alginate component of EPS. When monochloramine in the aqueous solution reaches the bacterial cell surface, the slow reaction with alginate-based EPS on the cell surface may limit monochloramine interaction with the cell membrane (Kouame & Haas, 1991). The lag time in the inactivation curves and nonsevere cell membrane damage in the FTIR results both support the hypothesis that monochloramine inactivation may be initiated through reactions at several sensitive sites on the bacterial surface. The presence of alginate EPS reduces the available reaction sites for monochloramine inactivation on the cell surface, and the slow penetration of monochloramine through the alginate EPS retards the protein inhibition. The decreased monochloramine demand witnessed for the high alginate EPS-producing mucA22 mutant indicates that the protective effect of capsular EPS against monochloramine is not attributed to disinfectant consumption by EPS, but instead it limits and delays accessibility to available reaction sites on the cell membrane for monochloramine. In contrast to the monochloramine inactivation, chlorine performs disinfection by fast oxidation of cellular material, which has been reported by many researchers (Gagnon et al., 2004). The high EPS-producing mucA22 excretes copious materials on the cell membrane that nonselectively react with these disinfectants, thus reducing available disinfectant before cell surface reactions occur. Considering these two different inactivation mechanisms, alginate EPS could limit microbial deactivation in two different ways, either acting as a disinfectant-demanding substrate or as a reaction-limiting material (reduced monochloramine penetration).

The results from fluorescent staining offered further support that capsular EPS provide a protective role for bacteria in the inactivation process, contributing to a VBNC state found in disinfected microorganisms (Rowan, 2004). The presence of capsular EPS necessitated longer times for bacterial inactivation by disinfectants. Limited PI penetration of the cells increases the presence of yellow- or orange-labeled cells (Phe et al., 2005; Virto et al., 2005), occurring more frequently for the mucA22 mutant strain than the other two strains (Fig. S5). Both a higher
viable rate and more intermediate state for the mucA22 indicate a greater possibility that this organism suffered from partial membrane injury and not from lethal membrane damage, when compared with the other two strains. An interruption of membrane permeability aided by capsular EPS could be a possible reason to explain the appearance of the intermediate-state organisms, which may maintain their capacity to regrow and cause public health problems.

To monitor the occurrence of membrane permeabilization and membrane damage, possible leakage of the intercellular material released from cells was measured at 260 nm (Samrakandi et al., 1997). Virto et al. (2005) reported that exposure of bacterial cells to chlorine in distilled water caused extensive damage to the cell membrane. However, the chlorine concentrations used were much higher (as high as 50 mg L\(^{-1}\)) than residual disinfectant concentrations in the water distribution systems. Our results (data not shown) suggest that leakage of intercellular material was not remarkable for the low disinfectant concentration used in this study. The results support that severe membrane damage or rupture is not a key event in the inactivation by chlorine and monochloramine at residual concentrations. Observations from other researchers hypothesized that more subtle events, such as enzyme activity or functional group deformation in the cell membrane, are involved in the bactericidal mechanism of free chlorine (Venkobachar et al., 1977). Functional group deformation in the cell membrane may alter membrane permeability and increase the probability of disinfectant entry into bacteria cells. To confirm the functional groups’ deformation, FTIR analysis was employed using both bacterial cells and extracted EPS. FTIR results revealed a decrease in IR absorbance for the C–O–C stretching vibrations of polysaccharides (1200–900 cm\(^{-1}\)), indicative of the cell wall peptidoglycan layer, and lipopolysaccharide outer leaflet was observed (Kansiz et al., 1999); however, the associated peak was not eliminated, potentially indicating nonsevere membrane damage.

Further analysis of FTIR provided evidence indicating the changes in chemical composition and concentration for both bacterial cells and extracted EPS occurred after exposure to disinfectants (Fig. 4). A higher reduction in absorbance intensity of protein and polysaccharide groups for mucA22 was observed, indicating that greater amounts of these components interacted with disinfectants. Comparing the three strains at the same inactivation threshold, infrared spectra show more polysaccharide and protein content react with the disinfectant for the mucA22 strain, which is attributed to reactions with capsular EPS. Macromolecules of bacterial EPS on the outer membrane possess active functional groups, including proteins and polysaccharides, which play a significant role in the interaction of the cell wall with the surrounding environment (Hong & Brown, 2006). Similarities in infrared spectrum when comparing bacterial cells and extracted EPS likely indicate that the spectra of the intact bacteria cells largely reflected the properties of the cell wall and cell wall-associated EPS. Therefore, the distinctive functional groups of capsular EPS and inner cell substances could be identified by subtracting the extracted EPS spectra from the bacterial cell spectra. In a study considering only whole-cell spectra, a decrease in infrared absorbance intensities for corresponding wave number ranges were observed after disinfection tests, providing information about deformation and reduction in characteristic functional groups in the cell membrane of Escherichia coli (Al-Qadiri et al., 2008).

In comparison with functional group interaction with the two tested disinfectants, it is clear that these disinfectants react very differently with cellular biomolecules (Fig. 5 and Fig. S3). Peak area reductions reveal that protein band reductions for each disinfectant are similar (c. 80% for all disinfectants). However, reductions in polysaccharide bands for monochloramine disinfectant are much less significant as compared to chlorine, whose polysaccharide reduction is comparable to protein reduction. This data confirm results obtained from disinfectant consumption and the extracted EPS spectra (Fig. 4b).

In summary, the results from this study provide a mechanistic understanding regarding the protective role of capsular EPS in response to chlorine and monochloramine. In-depth evaluation of the capsular EPS from both whole-cell and extracted EPS analyses revealed that capsular EPS may enhance bacterial survival in two distinct ways. In the presence of potent oxidative disinfectants, chlorine, capsular EPS at the surface of the cell membrane consumes disinfectant residuals, thus reducing their availability and efficacy for microbial inactivation. Alternatively, alginate EPS had minimal monochloramine demand, although the increased presence of alginate EPS was shown to reduce the efficacy of monochloramine, which showed high reactivity with proteins. Thus, the alginate EPS obscured disinfectant reactive sites on cell surface and retarded bacterial membrane interaction with monochloramine. In addition to EPS reactivity with different disinfectants, capsular EPS on cell membrane appeared to reduce membrane permeabilization by oxidative disinfectants, which was observed by functional group deformation. Functional moieties comprising the bacterial cell and capsular EPS were not eliminated, but were instead reduced in magnitude upon exposure to disinfectants. This suggests that the extensive membrane damage does not occur, given low concentrations of chlorine-based disinfectant residuals in water distribution.
systems. The combined results supported that capsular EPS, either by consuming disinfectant or limiting direct cell membrane access, provide a protective role for bacterial cells against regulatory residual disinfectants by reducing membrane permeabilization.

Acknowledgements

This research was supported by the US Geological Survey (Project Number: 2009OH89B) and the National Science Foundation (CBET0933288). And we greatly appreciate Dr Mau-yi Wu for his input.

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

*Fig. S1.* SEM images of *Pseudomonas aeruginosa* cells. (a) algT(U); (b) PAO1; (c) mucA22.

*Fig. S2.* FTIR spectra of monochloramine treated bacterial cells.

*Fig. S3.* Transformed FTIR spectra of monochloramine treated bacterial cells.

*Fig. S4.* Inactivation kinetics of EPS removed *Pseudomonas aeruginosa* cells by (a) chlorine and (b) monochloramine.

*Fig. S5.* LIVE/DEAD stained mucA22 cells after chlorine treatment by (a) chlorine and (b) monochloramine.