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Extraction and Analysis of Extracellular Polymeric Substances: Comparison of Methods and Extracellular Polymeric Substance Levels in *Salmonella pullorum* SA 1685

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Abstract

Extracellular polymeric substances (EPS) production and composition for *Salmonella pullorum* SA 1685 exposed to artificial groundwater (AGW) has been examined utilizing three EPS extraction methods: lyophilization, ethanol, and sonication. Experiments were carried out to evaluate the robustness of three EPS extraction methods and the sensitivity of each to subtle changes in solution ionic strength (IS) and duration of exposure. EPS extraction and analysis was conducted via sugar and protein analyses using the phenol sulfuric acid and Lowry methods, respectively, after 0-, 6-, 12-, 18-, and 24-h incubation times in AGW with $10^{-2.5}$, 10^{-2}, and $10^{-1.5}$ M IS. Lyophilization and ethanol methods resulted in a greater amount of EPS extracted than the sonication method (mass of EPS/cell), yet these methods fluctuated to a greater extent in the total amount—or level—of EPS extracted under the various test conditions. Systematic comparisons and extensive statistical analyses were conducted between the various experimental conditions. To our knowledge, this is the first study systematically comparing EPS extraction techniques utilizing *Salmonella*. As we investigated the relative EPS content in *Salmonella* SA1685 under conditions simulating groundwater, our results provide insight into the suitability of each method for detection of environmentally induced changes in bacteria suspended in simulated or real subsurface aquatic systems.

Key words: *Salmonella*; extracellular polymeric substances; extraction; sonication; bacterial starvation; cell lysis

Introduction

Bacteria have unique physical, chemical, and biological characteristics, one of which is the production of extracellular polymeric substances (EPS) (Ferris and Beveridge, 1985), which contributes to the degree to which the cells stick to surfaces (Costerton *et al.*, 1978; Frank and Belfort, 2003; Tsuneda *et al.*, 2003) or each other (Flemming and Wingender, 2001; Eboigbodin and Biggs, 2008; Gao *et al.*, 2008). EPS is a complex polymer matrix comprised of polysaccharides (Sutherland and Kennedy, 1996; Cescutti *et al.*, 1999), proteins (Fang and Jia, 1996; Veiga *et al.*, 1997), and nucleic acids (Zhang *et al.*, 1999) on the outside of the cell membrane (Wloka *et al.*, 2006). The EPS can be classified as either bound EPS or free EPS (Nielsen and Jahn, 1999). The bound EPS, also known as capsular EPS, is loosely bound around the cell surface, whereas the free or soluble EPS is exuded from the cell and released into the surrounding media (Nielsen and Jahn, 1999). Most cells produce EPS for protection (Sutherland, 1997; Wozniak *et al.*, 2003) and to release waste as part of metabolic processes (Morgan *et al.*, 1990).

EPS can serve an important role in cell adhesion and retention on mineral surfaces, impacting the fate and transport of micro-organisms in groundwater environments (Frank and Belfort, 2003; Tsuneda *et al.*, 2003; Walker *et al.*, 2005; Liu *et al.*, 2007). Recent literature demonstrates the ability of EPS to enhance cell retention on the surfaces through the formation of a conditioning film outside of the cells (Frolund *et al.*, 1996b; de Kerchove and Elimelech, 2007). The literature also suggests that the complexity and the gel forming properties of the EPS enhance the interaction of the cells with the other components around them (Watanabe *et al.*, 1999; Frank and Belfort, 2003; Olofsson *et al.*, 2003; Walker, 2005). In the groundwater environment, this enhanced level of interaction of cells with mineral surfaces or other cells leads to greater removal from the pore water and reduced transport in the subsurface (Liu *et al.*, 2007).

There are numerous methods that have been developed for the extraction of EPS, each appropriate under different scenarios. The requirements of a good EPS extraction method

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should include: (1) causing minimal cell lysis; (2) not disrupting or altering the characteristics of EPS; and (3) the ability to release and collect all of the EPS biopolymers (Nielsen and Jahn, 1999). In the literature, methods have been reported to achieve the task of separating EPS from the cell (Wuertz *et al.*, 2001; de Brouwer *et al.*, 2002; Liu and Fang, 2002; Comte *et al.*, 2006), and they can be divided into three categories: physical methods, chemical methods, and a combination of the two, for extracting the free and bound EPS from the cell (Nielsen and Jahn, 1999). Physical methods involve the techniques of separation with physical forces, for example, centrifugation, dialysis, filtration, sonication (King and Forster, 1990), cation exchange (Frolund *et al.*, 1996a), and heating (Karapanagiotis *et al.*, 1989; Morgan *et al.*, 1990). Chemical methods utilize chemical reagents, like ethylenediaminetetraacetic acid (EDTA), formaldehyde (H_2CO), sodium hydroxide (NaOH), and ethanol (C_2H_5OH) to fulfill the objective of separating EPS from the cells (Liu and Fang, 2002). In the literature, the chemical methods have been reported to yield more than physical methods; however, with higher probability of contamination from the reagent or cell lysis (Nielsen and Jahn, 1999). Although physical methods usually yield less than chemical methods, the fact that they usually have minimal contamination and cell lysis also makes them effective approaches for EPS extraction (Comte *et al.*, 2006). The combination of both chemical reagents and physical forces renders the method more reproducible and effective because a similar yield can be obtained without excessive contamination and cell lysis caused by the reagents (Nielsen and Jahn, 1999). To date, there have been limited studies on the extraction of EPS from *Salmonella*, and comparisons of extraction methods with this important organism have not been reported (Haznedaroglu *et al.*, 2009).

There are many techniques available for analyzing and quantifying the components of EPS, including colorimetric methods (Lowry *et al.*, 1951; Dubois *et al.*, 1956), Fourier transform infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), total organic carbon (TOC), high-performance size-exclusion chromatography (HPSEC) (Omoike and Chorover, 2004), gas chromatography-mass spectrometry (GC-MS), proton NMR (Hydrogen-1 NMR, or ^{1}HNMR) (Harding *et al.*, 2003), high-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) (Garrote *et al.*, 1992), atomic force microscope (AFM) (Ducker *et al.*, 1991; van der Aa and Dufrene, 2002), DNA assays (Wingender *et al.*, 2001), infrared spectroscopy (IR spectroscopy), and glucose-6-phosphate dehydrogenase (G6PDH) (Lessie and Vanderwy, 1972; Frolund *et al.*, 1996a; Nielsen and Jahn, 1999). To quantify polysaccharides content in EPS, one traditional method is the phenol sulfuric acid (PSA) method (Dubois *et al.*, 1956). Lowry *et al.* (1951), Bradford (1976), and Smith *et al.* (1985) methods are the typical approaches for bulk protein content quantification. These established colorimetric methods have been widely used, are easy to perform, and have been proven reliable. To ensure only extracellular content is evaluated, the extent of cell lysis also needs to be compared, even though extraction should be able to collect the EPS and keep the cells intact (Nielsen and Jahn, 1999). Typically, this is performed with an assay identifying presence of inner cell constituents (Lessie and Vanderwy, 1972; Frolund *et al.*, 1996a; Nielsen and Jahn, 1999; Wingender *et al.*, 2001).

Salmonella is a food-borne and water-borne pathogenic bacteria that has caused a considerable number of outbreaks (Chalker and Blaser, 1988; Killalea *et al.*, 1996; Rabsch *et al.*, 2003; Patrick *et al.*, 2004; Voetsch *et al.*, 2004). In the United States alone, approximately 30,000 to 40,000 culture-confirmed cases of nontyphoidal *Salmonella* are reported every year to the Centers for Disease Control and Prevention (CDC) (Chalker and Blaser, 1988; Mead *et al.*, 1999; Voetsch *et al.*, 2004). Common contaminated food includes meat-related food like processed food with poultry, egg, and ground beef (Williams *et al.*, 1968; Wray and Sojka, 1977). *Salmonella*, a Gram-negative bacterium, can also be transmitted through water (Coffey *et al.*, 2007). To study the fate of these organisms, the nature of the EPS must be characterized. To understand the extent to which the EPS production and composition responds to various environmental conditions, a combination of bound EPS extraction and analysis methods were conducted on cells exposed to relevant environmental conditions artificial ground water (AGW) at varying ionic strengths. Systematic comparisons and statistical analyses were made between the three methods, three IS, and four different periods of time in which cells were exposed to the various experimental solution chemistry conditions, in regard to total sugar content, protein content, and the sugar to protein ratio in the extracted EPS. The objective of this work is to quantitatively compare three EPS extraction methods in their capacity to (1) extract EPS from *Salmonella* and (2) resolve the subtle changes in EPS composition in response to three different ionic strengths and four periods of time in which cells were exposed to the various solution chemistry conditions.

Experimental Protocols

Cell selection and preparation

To perform this research, a model strain *Salmonella enterica* serovar pullorum (SA 1685) was used. This strain is an avian pathogen known to affect poultry (Shivaprasad, 2000; Berchieri *et al.*, 2001). Related *Salmonella* species are notorious, having caused numerous outbreaks in both humans (Mermin *et al.*, 2004; Patrick *et al.*, 2004) and animals (Snoeyenbos, 1991; Mermin *et al.*, 2004). This particular strain was also selected as it is a nonflagellated, nonmotile (Uzzau *et al.*, 2000), EPS-producing strain (Haznedaroglu *et al.*, 2009). Prior to each experiment, a preculture was prepared by incubating a sample of SA1685 cells at 37°C overnight in Luria-Bertani (LB) broth (Fisher Scientific, Fairlawn, NJ). The overnight preculture was utilized to inoculate a fresh culture (1:100 v/v), which was incubated at 37°C until reaching mid-exponential growth stage (3.5 h). Subsequently, cells were harvested by centrifugation (5804R; Eppendorf, Hamburg, Germany) equipped with a fixed-angle rotor (F-34-6-38; Eppendorf) for 15 min at 4°C and 3,700×*g*. The sample was decanted, the pellet resuspended in 10^{-2} M KCl, and the centrifugation repeated. This rinsing step was repeated two times, and the final pellet was resuspended in 1 mL of 10^{-2} M KCl and utilized as a stock solution for the following experiments.

The stock solution concentration was measured for each harvested culture. This was done utilizing a cell counting chamber (Burker Turk Superior Marienfel) and light microscope (Fisher Scientific Micromaster). Cell concentrations were $3.44 \pm 1.64 \times 10^{10}$ cells/mL in the stock solutions.

Exposure of model organism to groundwater conditions

Following the harvesting steps for SA 1685, the cells were exposed to a stress condition (varying AGW IS and time of exposure). Experiments were designed to simulate aquatic chemistry conditions existing in the subsurface environment. Cells were exposed to three representative IS of $10^{-2.5}$, 10^{-2}, and $10^{-1.5}$ M AGW solution (a liter of 10^{-2} M AGW contains 0.1071 g $CaCl_2 \cdot 2H_2O$, 0.0744 g $CaSO_4 \cdot 2H_2O$, 0.0595 g KNO_3, 0.1071 g $NaHCO_3$, 0.1042 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.1786 g $MgSO_4 \cdot 7H_2O$ (Bolster *et al.*, 1999 with slight modification)) for durations of 6, 12, 18, and 24 h. Cell concentrations were maintained at 10^8 cells/mL in the AGW cell suspensions. During this exposure time cell suspensions were gently shaken at 3.5 rpm (VWR OS-500 Analog Orbital Shaker; West Chester, PA) at room temperature (22–25°C). All reagents utilized were ACS grade (Fisher Scientific).

Extraction of EPS

To understand the extent to which the EPS production and composition responds to various environmental conditions, a combination of EPS extraction and analysis methods were conducted. For each exposure time as noted above, three different methods were utilized to separate EPS from the cell surfaces without lysing them. These methods were the lyophilization, ethanol (Azeredo *et al.*, 1999), and sonication methods (Liu *et al.*, 2007). Subsequently, the composition and content of the EPS was analyzed and evaluated as a function of the imposed stress.

The first two extraction methods, lyophilization and ethanol, are similar in nature, as both were initiated with resuspending the freshly harvested cell pellet in 10 mL 0.22% formaldehyde (ACS grade, Fisher Scientific) in 8.5% sodium chloride for 2 h in a 4°C incubator. Following the exposure to formaldehyde, the suspension was centrifuged (3,700×*g*, 4°C, 15 min) and the resulting pellet containing the EPS was resuspended in 10 mL deionized (DI) water (Millipore, Billerica, MA). Then the suspension was centrifuged again (3,700×*g*, 4°C, 15 min) to rinse away any remaining cellular material (non-EPS), the pellet was collected, its weight was measured, and 50 mL DI water per gram of pellet was added to resuspend the pellet. This solution was sonicated for 3 min (460/H Elma Transsonic Lab-Line Instruments, Melrose Park, IL) to purify the EPS, and final samples were centrifuged once more at 3,700×*g*, 4°C for 15 min (Eppendorf Centrifuge 5804R) to collect the purified EPS in the pellet.

The lyophilization and ethanol extraction methods deviated in the next and final stage of pellet handling. In the next stage of the lyophilization method, the pellet in 50 mL centrifuge tube was then placed in the freezer (−80°C) for 15 min. Finally, the sample was placed in a lyophilizer for a minimum of 6 h at −60°C and at a low pressure setting (~60 mTorr) (VirTis lyophilizer; Gardiner, NY). After 6 h of lyophilization, the pelleted EPS was resuspened in 10 mL DI water (Millipore), mixed by vortexing, and stored for later analysis.

The final step of the ethanol method was to resuspend the pellet in 5 mL 10^{-2} M KCl and 10 mL pure and cold ethanol and to incubate overnight at 4°C, resulting in precipitation of the EPS. After the incubation, the solution was then centrifuged (3,700×*g*, 4°C, 20 min), decanted, and 10 mL DI water (Millipore) added to the final pellet and resuspended for later analysis.

The third and final method for EPS extraction was the sonication method (Liu *et al.*, 2007). This method utilizes a cell disruptor machine (VirSonic Digital 600; VirTis) to remove EPS from cell surfaces. This was done by taking the freshly harvested cell pellet, resuspending it in 10 mL of 10^{-2} M KCl, exposing it to the stress condition of choice, and then sonicating the suspension (10 mL of 10^{-2} M KCl) for total of 20 s, with four runs of 5 s on, and three 5 s pauses in between the sonication in 3.5 Hz power level. The difference between this cell disruptor and the water bath sonication method utilized in the other two methods was that the disruptor probe was placed directly into the bacteria suspension to have a higher degree of impact on the cell. For consistency, 1 cm of the metal probe was immersed while conducting sonication. Afterward, the suspension was once again centrifuged at 4°C and 4,000×*g* for 20 min. Following this step, the cells remained in the pellet and EPS in the supernatant. Finally, the solution was filtered through a 0.22-μm filter (Millipore, Fisher Scientific) to ensure a cell-free, EPS suspension.

Evaluating extent of EPS production and composition

Once the EPS was extracted, the resulting EPS suspension (resuspended in 10 mL DI water) was evaluated for the total amounts of sugar and protein using previously developed methods. Specifically, the sugar quantification was performed with Phenol-Sulfuric Acid (PSA) method (Dubois *et al.*, 1956), and the protein was quantified using the Lowry method (Lowry *et al.*, 1951). The PSA method involved adding 50 μL 80% (w/w) phenol solution (Fisher Scientific), followed by 5 mL of highly concentrated sulfuric acid 95.5% (Fisher Scientific) to 2 mL of resuspended EPS solution. The phenol, acid, and EPS suspension was incubated at room temperature (22–25°C) for 10 min, followed by incubation in a water bath (Lab-Line Instruments) for 20 min at 25–30°C. After the incubation steps, the dark yellow to brown color of the solution required an additional 4 h for stabilization, and this was done by leaving the suspension at room temperature. Final measurement of the suspension was done spectroscopically (Biospecmini Shimadzu Corp., Kyoto, Japan) at 480 nm with Xanthan gum as a standard.

Total protein amount quantification was performed with the Lowry method using bovine serum albumin (BSA; 1 mg/mL) (Fisher BioReagents, Fisher Scientific) as the standard and measured spectroscopically (BioSpec-mini) at a wavelength of 500 nm. Next, 0.3 mL of resuspended EPS solution (in 10 mL DI water) was put in a glass vial (20 mL in volume) and 1.5 mL alkaline copper reagent (made by combining 1 mL 2% $Na_2C_4H_4O_6$, 1 mL 1% $CuSO_4 \cdot 5H_2O$, and 98 mL 2% $NaCO_3$ in 10^{-1} M NaOH) followed by the addition of 75 μL Folin reagent (Folin and Ciocalteu's Phenol Reagent, MP Biomedicals, LLC, Germany). The subsequent mixture was incubated at room temperature for 30 min and measured in the spectrometer at 500 nm. The absorbance reading was compared to the standard curve for BSA to determine the concentration of protein in each EPS sample.

Further cell characterization

A cell lysis test was performed by measuring the amount of G6PDH (Lessie and Vanderwy, 1972; Frolund *et al.*, 1996a;

Nielsen and Jahn, 1999). The kit consisted of two reagents, G6PD R1 reagent (NADP 1.5 mM, maleimide 12 mM, buffer, stabilizer, and lysing agent), and G6PD R2 reagent (glucose-6-phosphate 1.05 mM, buffer, magnesium salt, and sodium azide added as preservative) (Pointe Scientific Inc., Canton. MI). One milliliter of R1 reagent was added to 0.5 mL of resuspended EPS solution (in 10 mL DI water) and incubated at room temperature for 5–10 min. Then 2 mL of R2 reagent was added and the mixture was then incubated in water bath for 5 min. After the addition of R2 and water bath, an absorbance reading was immediately taken (340 nm) and again after exactly 5 min. The change in absorbance, normalized by the 5 min time increment, was calculated as the rate of reaction occurring. This rate value was then compared to values on a standard curve comparing absorbance for known concentration of G6PDH, allowing for the severity of cell lysis under each cell condition and for all three extraction methods to be determined.

The cell lysis analysis was also performed in another test kit following Sigma protocol with chemicals from Sigma Aldrich (Sigma Chemical Co., St Louis, MO). The chemicals include glycylglycine buffer, G 6-P, β-NADP, and $MgCl_2$. After mixing the buffer, G 6-P, β-NADP, $MgCl_2$, and the extracted EPS solution the absorbance was recorded immediately and after 5 min (time = 0 and 5 min). G6PDH enzyme solution was applied as concentration standards.

Similar to the cell lysis analysis, viability tests were also performed under each experimental condition using the Live/Dead BacLight kit (L-7012; Molecular Probes, Eugene, OR) and an inverted fluorescent microscope (IX70, Olympus, Japan) with a red/green fluorescence filter set (Chroma Technology Corp., Brattleboro, VT). The Live/Dead BacLight kit could only be performed before the starting of the extraction process, as it requires whole cells. Therefore, analysis of viability was conducted immediately after whole cells were exposed to AGW for the particular test condition. The G6PDH test could be performed after all of the EPS extraction steps, and indicated the cumulative cell membrane breakage through the measuring of inner cell enzyme concentration. Therefore, both methods were used.

Statistical analysis of results

Analysis of variance (ANOVA) was performed to determine whether the three extraction methods tested (lyophilization, ethanol, and sonication) yielded significantly different values of EPS sugar and protein content under the various combinations of IS ($10^{-2.5}$, 10^{-2}, and $10^{-1.5}$ M AGW) and starvation period (6, 12, 18, and 24 h) used to represent a range in environmental conditions. ANOVAs were performed using PROC MIXED in SAS (SAS Institute, 2003) and individual mean comparisons were based on Least Significant Differences (LSD) at $p \leq 0.05$. The experimental design was a split-plot design with the main unit having a two-way factorial treatment structure (IS and exposure time) and subunit being extraction method. The main unit had a randomized complete block design with two replicate blocks.

Pearson's correlation coefficients were calculated to determine if EPS protein concentrations were significantly correlated with EPS sugar concentrations for each of the three removal methods. In addition, multiple regression analysis was used to determine whether IS and starvation period had a significant effect on EPS sugar and protein concentrations for the three different extraction methods. All statistical analyses were performed using SAS version 9.1 (SAS Institute, 2003). Probability values less than 0.05 were considered statistically significant.

Results and Discussion

The EPS composition of *Salmonella* SA1685 cells were determined using a combination of extraction and analysis methods. The response to various environmental conditions, notably IS of AGW and time of exposure to said conditions was evaluated through the quantification of EPS yield and sugar and protein levels. The results of these analyses are

Table 1. Sugar Content of Extracellular Polymeric Substances Extracted by the Lyophilization, Ethanol, and Sonication Methods

		Sugar content (mg/10^{11} cells)†		
IS (M)[1]	*Time (h)*	*Lyophilization*	*Ethanol*	*Sonication*
10^{-5}	0*	3.46 ± 0.75 a	3.13 ± 1.42 a	2.34 ± 0.61 a
$10^{-2.5}$	6	2.64 ± 0.07 a	3.02 ± 0.20 a	1.00 ± 0.09 a
	12	2.24 ± 0.35 a	3.23 ± 0.32 a	1.56 ± 0.11 a
	18	3.86 ± 1.28 a	2.95 ± 0.78 a,b	1.04 ± 0.03 b
	24	3.48 ± 0.27 a	3.55 ± 0.16 a	2.24 ± 0.16 a
10^{-2}	6	4.09 ± 1.84 a	2.30 ± 0.40 a,b	1.21 ± 0.12 b
	12	3.57 ± 0.83 a	2.94 ± 0.89 a	2.36 ± 0.71 a
	18	5.53 ± 1.40 a	4.40 ± 1.52 a,b	3.02 ± 0.61 b
	24	2.44 ± 0.44 a	3.40 ± 0.96 a	1.57 ± 0.37 a
$10^{-1.5}$	6	2.57 ± 0.48 a	1.62 ± 0.09 a	0.71 ± 0.06 a
	12	3.10 ± 0.48 a,b	3.66 ± 0.63 a	0.71 ± 0.02 b
	18	4.62 ± 1.07 a	3.57 ± 0.37 a	2.93 ± 0.21 a
	24	7.88 ± 2.47 a,b	8.42 ± 3.99 a	5.75 ± 0.18 b

*Solution of deionized water only, not supplemented with artificial groundwater (AGW).
†Letters in the same row (a and/or b) indicate a statistically significant difference based on Least Significant Differences (LSD) means separation.
IS, ionic strength.

Table 2. Protein Content of the Extracellular Polymeric Substances Extracted by the Lyophilization, Ethanol, and Sonication Methods

		Protein content ($mg/10^{11}$ cells)†		
IS (M)	*Time (h)*	*Lyophilization*	*Ethanol*	*Sonication*
10^{-5}	0*	128.29 ± 17.59 a	174.20 ± 45.11 a	60.79 ± 23.16 b
$10^{-2.5}$	6	89.13 ± 5.97 a,b	112.46 ± 29.62 a	23.48 ± 2.02 b
	12	51.26 ± 13.05 a	77.86 ± 1.96 a	34.17 ± 6.99 a
	18	99.13 ± 43.27 a	76.22 ± 15.79 a,b	18.96 ± 0.32 b
	24	67.71 ± 8.31 a	71.80 ± 5.61 a	41.53 ± 1.78 a
10^{-2}	6	111.31 ± 25.86 a	75.86 ± 8.65 a,b	24.13 ± 1.95 b
	12	81.18 ± 21.84 a	391.11 ± 136.64 b	42.84 ± 12.15 a
	18	172.85 ± 26.41 a	137.86 ± 50.04 a,b	76.33 ± 24.78 b
	24	57.16 ± 13.56 a	83.29 ± 22.58 a	31.31 ± 4.29 a
$10^{-1.5}$	6	75.90 ± 17.20 a	51.32 ± 6.43 a	5.02 ± 1.23 a
	12	130.34 ± 26.69 a	175.21 ± 32.67 a	14.72 ± 1.40 b
	18	105.44 ± 11.29 a	89.99 ± 5.18 a	64.97 ± 5.36 a
	24	149.98 ± 41.31 a	143.08 ± 73.19 a	83.30 ± 3.31 a

*Solution of deionized water only, not supplemented with AGW.
†Letters in the same row (a and/or b) indicate a statistically significant difference based on LSD means separation.

shown in Tables 1–3. The relative performances of the three extraction methods in capturing the EPS trends under the range of environmental conditions tested have been examined by statistical analysis. Letters in the same row (a and/or b) indicate a statistically significant difference between the three methods based on LSD means separation in Tables 1–3.

Based on the ANOVA results, extraction method had a significant effect ($p < 0.001$) on measured EPS sugar and protein content (Table 4). IS and exposure time also had a significant effect for all three extraction methods combined indicating that EPS protein and sugar content varied depending on the experimental conditions. For EPS sugar, all interaction terms with method of extraction were not statistically significant indicating the *F* test for extraction method (averaged over all IS and exposure time) is accurate. For the protein data the interaction terms were significant; however, because the *F* values were much lower for these interaction terms than the main effect for method, the *F* test for extraction method obtained for EPS protein is also accurate. Further discussion on the experimental trends and outcomes of our statistical analyses are discussed below.

Comparison of the EPS yield

The three extraction methods, lyophilization, ethanol, and sonication, were compared based upon the sugar and protein amount combined together as total EPS extracted. Between the lyophilization and ethanol methods, the total mass of the extracted EPS pellet—the mass of EPS measured before the final purification step by freeze drying or ethanol exposure—did not change significantly regardless of IS and exposure time (not significant with student's paired *t*-test, $p > 0.1$). Typically, the extracted pellet mass prior to the final purification step weighed approximately 0.5 g; however, this was a measured wet weight consisting not only of the EPS pellet but also residual DI water. Hence, further analysis focused not on the

Table 3. Sugar-to-Protein Ratio of the Extracellular Polymeric Substances Extracted by Lyophilization, Ethanol, and Sonication Methods

		S/P Ratio†		
IS (M)	*Time (h)*	*Lyophilization*	*Ethanol*	*Sonication*
10^{-5}	0*	0.027 ± 0.003 a	0.019 ± 0.010 a	0.041 ± 0.010 b
$10^{-2.5}$	6	0.030 ± 0.002 a,b	0.028 ± 0.005 a	0.043 ± 0.003 b
	12	0.045 ± 0.005 a	0.041 ± 0.004 a	0.048 ± 0.013 a
	18	0.041 ± 0.005 a,b	0.038 ± 0.002 a	0.055 ± 0.001 b
	24	0.051 ± 0.004 a	0.050 ± 0.003 a	0.054 ± 0.002 a
10^{-2}	6	0.035 ± 0.009 a	0.030 ± 0.003 a	0.050 ± 0.003 b
	12	0.044 ± 0.003 a	0.008 ± 0.001 b	0.055 ± 0.003 a
	18	0.032 ± 0.004 a	0.032 ± 0.002 a	0.041 ± 0.007 a
	24	0.043 ± 0.006 a	0.041 ± 0.001 a	0.049 ± 0.005 a
$10^{-1.5}$	6	0.034 ± 0.002 a	0.032 ± 0.003 a	0.153 ± 0.057 b
	12	0.024 ± 0.002 a	0.021 ± 0.003 a	0.048 ± 0.006 b
	18	0.043 ± 0.006 a	0.040 ± 0.003 a	0.045 ± 0.004 a
	24	0.053 ± 0.009 a	0.060 ± 0.006 a,b	0.069 ± 0.003 b

*Solution of deionized water only, not supplemented with AGW.
†Letters in the same row (a and/or b) indicate a statistically significant difference based on LSD means separation.

Table 4. Analysis of Variance Summary Table for Sugar and Protein Content of Extracellular Polymeric Substances

	Sugar		*Protein*	
Effect	F *value*	p	F *value*	p
Method	14.0	<0.001	30.1	<0.001
IS	5.4	0.0089	7.50	0.0019
Time	10.1	<0.001	4.75	0.0068
Method×IS	0.24	0.92	2.56	0.055
Method×Time	0.55	0.77	6.16	<0.001
IS×Time	7.83	<0.001	5.65	<0.001
Method×IS×Time	0.37	0.97	3.14	0.0038

EPS pellet mass, but rather emphasized and reported the sum of sugar and protein content as the total EPS extracted (EPS yield).

For a given IS and exposure time, we observed differences in the yield of the EPS pellet extracted by the three different methods. In particular, the lyophilization and ethanol methods always yielded higher values of sugar and protein content than the sonication method. For example, the lyophilization and ethanol methods, on average, yielded 2.4 and 2.2 times more sugar, respectively, than the sonication method. For protein content, the lyophilization and ethanol methods yielded, on average, values 4.2 and 4.6 times, respectively, more than the sonication method. For several combinations of IS and exposure time, the values obtained with sonication were significantly different than those obtained with the lyophilization and/or ethanol extraction methods; of note, when EPS data were averaged over IS or time and were compared, the sonication method almost always yielded values significantly lower than the other two methods (data not shown). On the other hand, differences between the ethanol and lyophilization methods were not statistically significant under any of the environmental conditions tested (Tables 1 and 2). The lower observed EPS values obtained with the sonication method was attributed to this method being purely a physical extraction method; whereas, the lyophilization and ethanol methods were similar in their physical and chemical approach with only a difference in the last purifying step (see earlier). This finding is consistent with others who have reported lower yields with physical extraction methods (Nielsen and Jahn, 1999; Liu and Fang, 2002; Comte *et al.*, 2006). For example, Comte et al. found that after comparing eight methods of extraction, including both chemical and physical methods, notably less EPS was extracted by physical methods than the chemical methods.

Comparison of the sugar and protein content as a function of extraction method

When comparing data presented in Table 1 and displayed in Fig. 1a–c, it was observed that there was no notable difference between extraction methods in sugar content with changing solution chemistry (IS), as all three methods produced a fairly similar trend. There was an upward trend in sugar content as a function of exposure time observed in all three methods, particularly for cells exposed to the highest IS solution ($10^{-1.5}$ M). Over increasing periods of exposure, the

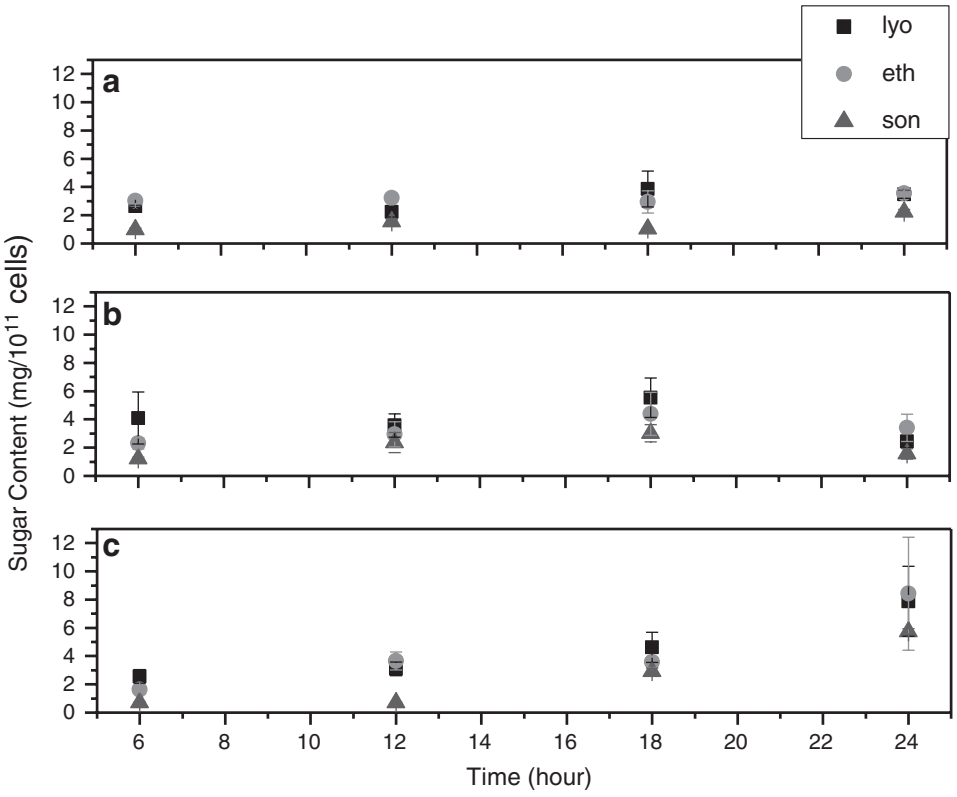

FIG. 1. Sugar content as a function of exposure time normalized by cell concentration. (**a**) $10^{-2.5}$ M, (**b**) 10^{-2} M, (**c**) $10^{-1.5}$ M. lyo, lyophilization; eth, ethanol; son, sonication.

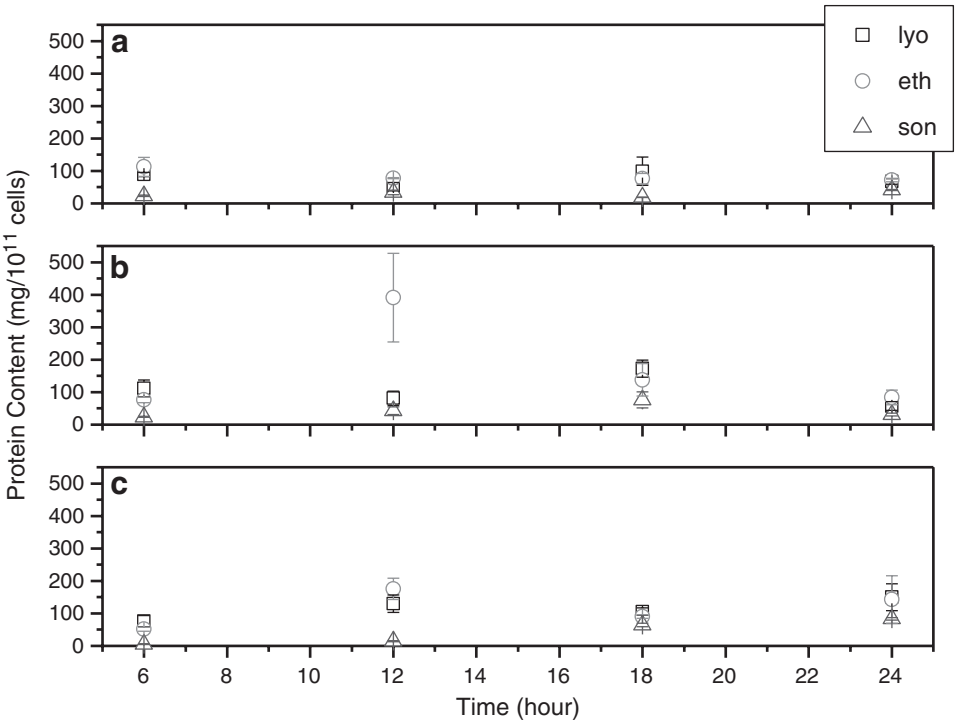

FIG. 2. Protein content as a function of exposure time normalized by cell concentration. (**a**) $10^{-2.5}$ M, (**b**) 10^{-2} M, (**c**) $10^{-1.5}$ M.

effect of solution chemistry (IS) became more evident, in that the overall increase in the amount of sugar as a function of exposure time was more pronounced at the higher IS conditions tested.

The extracted protein content is presented in Fig. 2, and Table 2, as a function of exposure time to AGW. There was no clear relationship between protein content, time, and IS (Fig. 2). The changes in protein content as a function of exposure time for all extraction methods were minor. When comparing the three methods' sensitivity to time, as shown in Fig. 2, all three followed a fairly similar trend for protein. The bulk of the comparisons were insignificant (24 of 39); however, the extraction methods were more sensitive to various environmental conditions (IS and duration of exposure) for EPS protein than sugar content. There was one outlying data point, for the ethanol sample after 12-h exposure to AGW (Table 2 and Fig. 2). This data point suggests an increase in protein after 12 h, but as the other two methods indicated, their protein levels were not changing significantly at the same condition (12 h); hence, this data point is considered an anomaly.

Further evaluation of the extraction methods was conducted to identify any correlation between extraction methods and corresponding protein and sugar levels. There were strong correlations between EPS sugar and protein content as measured by the sonication ($r^2 = 0.92$; $p < 0.001$) and lyophilization ($r^2 = 0.78$; $p = 0.003$) methods. For the ethanol extraction method, however, the correlation between EPS sugar and protein content was not significant ($r^2 = 0.12$, $p = 0.71$).

An alternative presentation of the data is shown in Supplementary Figs. S1–6. In Supplementary Figures S1–3, the EPS sugar and protein content extracted are presented as a function of exposure time and IS for each method. Multiple linear regressions performed on data from all 12 treatments showed significant increases in EPS content with increasing exposure time for sugar content as measured with the ethanol (slope = 0.15; $p = 0.031$) and sonication (slope = 0.12; $p = 0.035$) extraction methods and for protein content as measured with the sonication extraction method (slope = 2.1; $p = 0.041$). No significant correlations, however, were observed with ionic strength. Supplementary Figures S4–6 are more comprehensive, presenting the EPS sugar (part a) and protein (part b) content as a function of exposure time and ionic strength for each extraction method. These figures offer a different visual presentation of the data allowing individual trends with each method to be compared.

Comparisons utilizing a sugar/protein ratio

The ratio of sugar to protein (sugar/protein ratio) was another parameter that allowed for comparisons between the sensitivity of the three different extraction methods and has the potential to provide considerable insight into the cell response to subtle changes in environmental conditions (Table 3). Utilizing this approach, differences were observed between EPS extraction methods with regard to the resulting sugar/protein ratio. However, the letters, a and b, indicate two thirds (26 of 39) of the differences between methods were statistically insignificant. This suggests that the sugar/protein ratio can provide additional insight into changes in EPS. As mentioned before, the sugar and protein content extracted by the sonication method had a stronger linear correlation than lyophilization and ethanol methods. Hence, we observed a more stable sugar/protein ratio from the sonication data than the other two methods.

Comparisons through cell lysis analysis

The extent of cell lysis was shown to be relatively insignificant for all three extraction methods in various environmental

conditions based upon both test kits and protocols as distributed by Sigma and Pointe Scientific (data not shown). The three methods had negligibly low levels of lysis, through the detection of G6PDH. Our results suggested the organisms' membranes remained intact up to the point of EPS extraction, as indicated by greater than 90% viability prior to extraction by BacLight test kit. During the following extraction processes by any of the three methods, only minimal lysis occurred as indicated by our detection protocols. Hence, it is assumed that quantifiable levels of protein and sugar, as reported in Tables 1–2 are constituents of the EPS and not cytoplasmic or membrane-based materials.

Summary

The ability to measure subtle changes in *Salmonella pullorum* SA 1685 cells' EPS content was highly sensitive to the choice of extraction method. Notably, the ethanol method had the highest EPS yield of the three, slightly greater than the lyophilization method and much more than sonication method in all conditions. The ethanol method results in the same general trends as the lyophilization method, but with higher variance. The sonication method may have a lower yield, but was a much faster process to conduct needing no more than 1 h vs. the lengthy overnight steps involved in the other two methods.

The results of this study showed the sensitivity of three EPS extraction methods to various cell conditions (IS of AGW and exposure time). This provided insight on these three methods and helped to determine the capability of each in detecting environmental changes in simulated or real subsurface environments. Based upon the conditions investigated in this particular study, ANOVA evaluation deemed the three extraction methods were all valuable and capable of differentiating EPS trends; however, choice of extraction method has a significant effect on the subsequent measured EPS sugar and protein content. The method of extraction should be selected as appropriate for the particular goals of each individual investigation, keeping in mind that the degree of resolution of the EPS trends varies based upon the method selected. Outcome of this study is intended to serve as guidance for environmental scientists in their selection of EPS extraction and analysis protocols, indicating the choice of methods can clearly influence the results and needs careful consideration.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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