Chromium-contaminated soils threaten surface and groundwater quality at many industrial sites. In vadose zones, indigenous bacteria can reduce Cr(VI) to Cr(III), but the subsequent fate of Cr(III) and the roles of bacterial biofilms are relatively unknown. To investigate, we cultured *Pseudomonas putida*, a model organism for vadose zone bioremediation, as unsaturated biofilms on membranes overlaying iron-deficient solid media either containing molecular dichromate from potassium dichromate (Cr-only treatment) or with deposits of solid, dichromate-coated hematite (Fe+Cr treatment) to simulate vadose zone conditions. Controls included iron-deficient solid medium and an Fe-only treatment using solid hematite deposits. Under iron-deficient conditions, chromium exposure resulted in lower cell yield and lower amounts of cellular protein and carbohydrate, but providing iron in the form of hematite overcame these toxic effects of Cr. For the Cr and Fe+Cr treatments, Cr(VI) was completely reduced to Cr(III) that accumulated on biofilm cells and extracellular polymeric substances (EPSs). Chromium exposure resulted in elevated extracellular carbohydrates, protein, DNA, and EPS sugars that were relatively enriched in N-acetyl-glucosamine, rhamnose, glucose, and mannose. The proportions of EPS protein and carbohydrate relative to intracellular pools suggested Cr toxicity-mediated cell lysis as the origin. However, DNA accumulated extracellularly in amounts far greater than expected from cell lysis, and Cr was liberated when extracted EPS was treated with DNase. These results demonstrate that Cr accumulation in unsaturated biofilms occurs with enzymatic reduction of Cr(VI), cellular lysis, cellular association, and extracellular DNA binding of Cr(III), which altogether can facilitate localized biotic stabilization of Cr in contaminated vadose zones.

Chromium (Cr), an Environmental Protection Agency priority pollutant and suspected carcinogen (16), is a significant soil contaminant at many industrial sites, where it is used for manufacturing alloys and pigments, electroplating, leather tanning, wood preservation, and chemical synthesis (43). Chromium is also an important soil and water contaminant at several U.S. Department of Energy facilities (37, 68). The prevalent oxidation states of chromium in the soil environment are VI and III (38). Cr(VI) is toxic and poses a public health risk because of its mobility and the ease with which it can enter eukaryotic cells (10). The reduced state, Cr(III), however, is less soluble and is regarded as relatively harmless to humans. Thus, remediation efforts often focus on reduction-mediated immobilization of Cr (12).

Bacteria are often involved in the fate and transport of toxic metals in soil (35, 36, 50, 62). Both abiotic and biotic processes catalyze the reduction of Cr(VI) to Cr(III) in the natural environment (20), but bacterial reduction of Cr(VI) to Cr(III) can occur under nutrient-amended vadose zone conditions, which implies that biostabilization is a viable management option (42). In laboratory studies, Cr(VI) is actively transported into cells and then intracellularly reduced and effluxed as Cr(III) via the ChrA system (1). In *Pseudomonas* spp., Cr(III) then accumulates on the cell wall and outer membrane (63). Similar processes have been documented for other bacterial species and toxic metals (2, 29, 41, 60, 66). However, bacterium-metal interactions in the environment will also involve extracellular polymeric substances (EPSs) of bacterial biofilms, the form of bacterial growth most frequently occurring in nature (13, 14).

EPS facilitates nutrient and water retention, cell/substrate adhesion, cell-cell signaling, and protection of individual cells from chemical degradation or attack (5, 56, 57). Bacterial EPS can also bind contaminants (9, 59, 67, 72), which can lead to short-term stabilization or longer-term immobilization of metals (9). Metals also bind to cells in biofilms, as indicated by *Pseudomonas aeruginosa* biofilm cells accumulating relatively more Fe, Au, and La than planktonic *P. aeruginosa* (34). Cr(VI) binds to biofilms (11), but little is known about its specific interactions with either EPS or biofilm cells. Inferences can be drawn from *Enterobacter cloacae* grown in seawater, whereby Cr(VI) exposure resulted in increased EPS production and metal binding to cells and EPS (31). However, interactions of EPS and Cr may depend on the abundance and chemistry of EPS, which in turn varies with bacterial strain and nutrition.

EPS is composed of carbohydrates, protein, DNA, and sorbed abiotic constituents (56). Changes in the macromolecular composition of bacterial biofilms have been observed with alterations in carbon source (55), water availability (47), and exposure to toluene, a toxic hydrocarbon (48). Changes also
appear in response to toxic metals. For example, mixed-species Cd-exposed sulfate-reducing bacterial biofilms produced increased amounts of extracellular protein and carbohydrate (71). Chromium exposure also resulted in increased EPS production and changes in marine bacterial biofilm morphology under sulfate-reducing conditions (18). Also, an anaerobic bacterial consortia grown in a chemostat accumulated more EPS and cell lysis products upon exposure to Cr (3), but the association of Cr with either EPS or soluble microbial products was not quantified. While these examples suggest the possibility for similar effects in soil biofilms, EPS quantity and quality depend on growth conditions (56), which differ for saturated and unsaturated systems.

The purpose of this study was to quantify changes in the macromolecular composition of cellular and EPS fractions of unsaturated Pseudomonas putida mt-2 biofilms exposed to iron (Fe) and Cr and to relate such changes to Cr fates in the soil environment, where Cr is frequently bound to Fe oxides that are colonized by Cr- and Fe-reducing bacteria. Here, unsaturated biofilms refer to bacteria colonizing surfaces exposed to air and covered by only thin films of water (4, 26, 27, 53, 55), as would occur in vadose zones. We observed that Cr exposure increased EPS on a per-cell basis in membrane-cultivated unsaturated biofilms and that macromolecular chemistry varied with exposure to Cr. We also observed that Cr(VI) was completely reduced to Cr(III), which accumulated on cells and in EPS. Extraordinarily high amounts of extracellular DNA (eDNA) were also present, and additional studies were performed that confirmed an association between Cr(III) and eDNA which could contribute to Cr(III) biostabilization in vadose zones.

MATERIALS AND METHODS

Minerals and media. Hematite (Fe₂O₃) was synthesized according to standard methods (49), air dried, and autoclaved for 1 h. The synthesized hematite particles had a mean diameter of 32 μm and a mean specific surface area of 0.326 m² g⁻¹ as determined by a Malvern particle sizer (Malvern Instruments Ltd., Worcestershire, United Kingdom). To coat the hematite with dichromate, 0.20 g of hematite was equilibrated with 20 ml of a filter-sterilized (0.2 μm, Millipore, MA) 0.0147 g K₂Cr₂O₇ (0.05 mM, 0.2-ml wash) in 0.1 M H₂O, and stored at 20°C until analysis. Total carbohydrates for both the standards and biofilm samples were determined by the phenol-sulfuric acid method using glucose as the standard (17). Total protein was quantified using the Bradford method (6) with reagents from Bio-Rad (Hercules, CA). DNA was quantified by the Picogreen method (MOLECULAR PROBES, Eugene, OR) using calf thymus DNA as the standard. Glucosyl residues were derivatized, characterized, and quantified by gas chromatography/mass spectrometry (GC-MS) similarly to the method of York et al. (74) using a Hewlett-Packard 6890/5973 GC-MS instrument. Individual glucosyl residues were expressed on a mass-per-biofilm cell basis; their masses were also converted to glucose equivalents by applying the following formula:

\[
sugar mass \text{ mol wt sugar} = \text{moles C} \times \text{mol wt glucose}
\]

where mol wt is molecular weight.

In separate experiments using biofilms cultivated identically to the method described above, the amount of intracellular DNA (iDNA) (55) per cell was determined for each of the triplicated treatments. Cells and EPS were centrifuged (10 min × 1,500 rpm) and stored at −20°C for further analysis. The cell pellets were resuspended in 2.5 ml of 1.0 M NaOH and lysed by heating (80°C, 1 h). The lysed cell suspension was then neutralized with 2.5 ml of 1.0 M HCl and stored (−20°C) until analysis. Total carbohydrates for both the standards and biofilm samples were determined by the phenol-sulfuric acid method using glucose as the standard (17). Total protein was quantified using the Bradford method (6) with reagents from Bio-Rad (Hercules, CA). DNA was quantified by the Picogreen method (MOLECULAR PROBES, Eugene, OR) using calf thymus DNA as the standard. Glucosyl residues were derivatized, characterized, and quantified by gas chromatography/mass spectrometry (GC-MS) similarly to the method of York et al. (74) using a Hewlett-Packard 6890/5973 GC-MS instrument. Individual glucosyl residues were expressed on a mass-per-biofilm cell basis; their masses were also converted to glucose equivalents by applying the following formula:

\[
sugar mass \text{ mol wt sugar} = \text{moles C} \times \text{mol wt glucose}
\]
diluted 100×, and 150-μl aliquots were combined with 2.85 ml of 0.9% NaCl and 0.6 μl of SYBR Gold (Molecular Probes, Eugene, OR) in 3-ml foil-wrapped tubes. The suspension was incubated at room temperature in the dark for 20 min and then filtered through an Anodisc filter (25-mm diameter, 0.2-μm pore size; Whatman), which was then dried in the dark, mounted on a standard glass slide, and sealed with a coverslip. Cells were counted at a total magnification of ×1,000 using an Eclipse E800 epifluorescence microscope (Nikon) and a green fluorescent protein filter set (Chroma, Rockingham, VT). Three replicate slides were prepared for each biofilm. Cells were counted in 10 separate fields per slide, and counts were averaged across the fields for each slide.

Biofilm staining and ESEM imaging. For each of the treatments, three biofilms from separate petri dishes were examined using environmental scanning electron microscopy (ESEM). Prior to imaging, the biofilms were removed from the solid growth medium using sterile forceps and stained with a combination of ruthenium red, glutaraldehyde, osmium tetroxide, and lysine according to previous methods (J. H. Priester, A. M. Horst, L. C. Van De Werfhorst, J. L. Saleta, L. A. K. Mertes, and P. A. Holden, submitted for publication). The biofilms were imaged on a Peltier stage (5°C) in an FEI Co. XL30 FEG ESEM (Philips Electron Optics, Eindhoven, The Netherlands) operated in wet mode (∼4 Torr) at an accelerating voltage of 10 kV. Specimens were not conductively coated prior to imaging. A random-number-based scheme was used to select fields of view when acquiring biofilm images. Images (five per biofilm) used for assessing biofilm surface morphology and cell sizes were acquired at magnification ×2,500; biofilm porosity was estimated from triplicate images taken at magnification × 600.

Abiotic Cr desorption from hematite. One interest in this study was the possible involvement of biofilms in biotic desorption of Cr off of hematite–Fe. Such a process could be inferred only after accounting for the abiotic Cr desorption from hematite using solution chemistry identical to the biofilm culture conditions. Abiotic desorption of Cr from the Cr-coated hematite was tested by incubating (static, 30°C) various amounts (3.8 mg, 8.8 mg, 16.3 mg, and 24.9 mg) of dichromate-coated hematite in 5 ml of sterile liquid growth medium. After 72 h, the samples were centrifuged to separate particulate metals from soluble constituents. Soluble metals were quantified using ICP-AES after preparing samples in 10% (vol/vol) aqua regia as before. The concentration was evaluated and principles of mass balance invoked to infer the amount of Cr liberated from the solid growth medium using sterile forceps and stained with a combination of ruthenium red, glutaraldehyde, osmium tetroxide, and lysine according to previous methods (J. H. Priester, A. M. Horst, L. C. Van De Werfhorst, J. L. Saleta, L. A. K. Mertes, and P. A. Holden, submitted for publication). The biofilms were imaged on a Peltier stage (5°C) in an FEI Co. XL30 FEG ESEM (Philips Electron Optics, Eindhoven, The Netherlands) operated in wet mode (∼4 Torr) at an accelerating voltage of 10 kV. Specimens were not conductively coated prior to imaging. A random-number-based scheme was used to select fields of view when acquiring biofilm images. Images (five per biofilm) used for assessing biofilm surface morphology and cell sizes were acquired at magnification ×2,500; biofilm porosity was estimated from triplicate images taken at magnification × 600.

XANES. Cr K-edge XANES spectra were collected at the Stanford Synchrotron Radiation Laboratory beam line 11-2 under Stanford Positron Electron Asymmetric Ring 3 to determine the oxidation state of Cr. Triplicate samples for each treatment of biofilms, EPS, and cells were received overnight frozen and then thawed. All treatments were triplicated. The treatments included an inoculated and harvested, and EPS was separated from cells as described above. Enough biofilms were cultivated such that 20 biofilms were combined as 1 replicate in a treatment. All treatments were triplicated. The treatments included a control of undialyzed EPS in 0.9% NaCl, a test of dialyzed EPS in 0.9% NaCl, and dialyzed EPS in 0.9% NaCl treated with DNase I (Ambion, Austin, TX). An abiotic control for Cr transport through the dialysis tubing consisted of dialyzing 0.06 mg/ml Cr(III) [as Cr(NO3)3·6H2O] in 0.9% NaCl. All treatments were dialyzed so that unbound Cr would equilibrate across the dialysis tube, resulting in significantly lower final concentrations in the tubing. Dialysis was performed in Spectra/Por 1, flat-sheet 10-kDa membranes with a calculated weight of 6,000 to 8,000 (Fisher Scientific, Hampton, NH) using Nanopure water as the dialysis solution for 24 h. The water volume was approximately one liter and was changed after 12 h. Samples treated with DNase I were prepared by adding 100 μl of the enzyme (in Tris-HCl) to each sample and incubating for 1 h at 37°C. In this way, any Cr bound to eDNA would be liberated into solution upon DNA cleavage, and the relative role of eDNA in Cr binding could be inferred. Following dialysis, the contents of the dialysis tubes were sampled quantitatively into sterile tubes for drying. When the samples were completely dry, they were resuspended in 4 ml of 10% (vol/vol) aqueous regia and analyzed with ICP-AES for total chromium as before.

Data analysis. The content of macromolecules and metals in the EPS and cell fractions was normalized to the number of cells after determining the iDNA content on a per-cell basis for each treatment. The volumetric concentrations of metals in the biofilms were estimated by dividing the metal mass quantified for each biofilm by the biofilm volume. The biofilm volume (Vbio) was calculated using the numbers of cells per biofilm (no. cells), the cellular volume (Vcell), and the biofilm porosity as follows:

\[ V_{\text{bio}} = V_{\text{cell}} \times \text{no. cells} \times (1 - \text{porosity}) \]  

(2)

Biofilm porosity was estimated from each of three ×600-magnification ESEM images for each treatment in Photoshop 5.5 (Adobe, San Jose, CA) by dividing the area of space around cells by the total image area, assuming an equivalent projection into the third dimension (27).

Cellular dimensions were measured from ESEM images (×2,500) using Photoshop 5.5 for 10 randomly selected cells for each treatment. To quantify observed textural differences between biofilms, ESEM images were analyzed using the neighborhood variety statistic in Arcview GIS 3.2a (ESRI, Redlands, CA) as described before (Priester et al., submitted). The neighborhood variety statistic yields both maps of textural variety and histograms of pixel abundance versus variety. A rectangular 10-by-10 variety filter was used in Arcview. From the histogram data, a pixel-weighted mean variety statistic was calculated for each image. A relatively high mean variety results from texturally complex or rough samples, whereas a lower mean variety results when the specimen is smooth. Thus, the mean variety was used as a metric for comparing image textural differences between treatments which in turn resulted from differences in EPS abundance on the surfaces of biofilms.

All statistical analyses were performed using either SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL) or Microsoft Excel 2000 software. Means were compared by the Student t test. Where applicable, standard errors were propagated using accepted methods (38).
RESULTS

Yield. After 72 h, biofilm biomass was visibly greatest for the control treatment and least for the Cr and Fe+Cr treatments. The Fe treatment had intermediate biofilm growth. The total number of cells differed significantly only for the Cr treatment (Fig. 1), where cell counts were approximately one-third or less of those for the other treatments. In addition to total cell counts, iDNA is another proxy for biofilm yield (55). The control (0.024 ± 0.003 µg) and Fe (0.027 ± 0.005 µg) treatments yielded significantly greater amounts of iDNA per biofilm than the Cr (0.0057 ± 0.001 µg) treatment. The control treatment also yielded significantly more iDNA than the Fe/Cr treatment (0.013 ± 0.002 µg; P < 0.01). When compared to each other, the Fe/Cr and Fe treatments yielded similar amounts of iDNA per biofilm.

Other macromolecule contents in the biofilms also varied with treatment. The control biofilms contained significantly greater (P < 0.03) total (cellular plus EPS) protein (0.14 ± 0.006 mg) than those with the Cr treatment (0.09 ± 0.006 mg) and significantly more (P = 0.01) carbohydrate (0.07 ± 0.006 mg) than those with the Cr treatment (0.02 ± 0.006 mg). Total protein did not vary across the control, Fe (0.14 ± 0.006 mg), and Fe+Cr (0.13 ± 0.01 mg) treatments. The total carbohydrate in control biofilms was statistically greater (P = 0.01) than that in the Fe+Cr biofilm (0.03 ± 0.006 mg). The total carbohydrate for the Fe treatment (0.06 ± 0.01 mg) and the total protein for the Fe treatment did not differ from the respective macromolecular contents for the Fe+Cr treatment biofilms. Total DNA was highest for the Fe+Cr treatment (0.20 ± 0.006 µg) in comparison to either the control (0.11 ± 0.01 µg), the Fe (0.15 ± 0.006 µg), or the Cr (0.11 ± 0.01 µg) treatment.

Biofilm morphology. The biofilm diameters were between 5 and 10 mm, where the control treatment yielded the largest, and the Cr treatment the smallest, biofilms. The control treatment yielded thick, opaque biofilms with irregular edges. In contrast, the Cr and Fe+Cr biofilms were thin and semitransparent. Biofilms grown over hematite (Fe and Fe+Cr treatments) were pink. As indicated by the ESEM images (Fig. 2), individual cells in the control biofilms were easily distinguishable, while those in the biofilms exposed to the metals were not. Further, cells exposed to Fe were elongated (Table 1).

The mean variety of ESEM images, a measure of topographical irregularity on biofilm surfaces (J. H. Priester, A. M. Horst, L. C. Van De Werfhorst, J. L. Saleta, L. A. K. Mertes, and P. A. Holden, submitted for publication), was

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**FIG. 2.** Environmental scanning electron micrographs of *Pseudomonas putida* mt-2 unsaturated biofilms. Treatments are indicated in the figure and described in Materials and Methods. Scale bar = 10 µm.

**TABLE 1.** Average cellular dimensions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Length (µm ± SE)</th>
<th>Width (µm ± SE)</th>
<th>Aspect ratio (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.13 ± 0.05 a</td>
<td>0.67 ± 0.04 d</td>
<td>1.72 ± 0.08 g</td>
</tr>
<tr>
<td>Fe</td>
<td>1.44 ± 0.07 c</td>
<td>0.51 ± 0.02 e</td>
<td>2.82 ± 0.13 i</td>
</tr>
<tr>
<td>Fe+Cr</td>
<td>1.34 ± 0.05 e</td>
<td>0.52 ± 0.03 f</td>
<td>2.59 ± 0.09 i</td>
</tr>
<tr>
<td>Cr</td>
<td>1.02 ± 0.03 b</td>
<td>0.73 ± 0.02 d</td>
<td>1.40 ± 0.05 h</td>
</tr>
</tbody>
</table>

*Like letters in each column indicate differences that are not significant (t test, P > 0.05).*
highest for the control treatment (23.1 ± 0.7) and lowest for the Fe+Cr treatment (9.6 ± 0.2), indicating that the biofilms in the latter treatment were more smooth, possibly due to more EPS occupying divisions between cells. Images from the Fe and Cr treatments had intermediate values of mean variety (17.8 ± 0.5 and 16 ± 0.4, respectively). All differences in the mean variety across treatments were significant (P < 0.04).

**Macromolecular composition.** When macromolecular composition was expressed on a per-cell basis, total protein was highest in the Cr-exposed biofilms, but total biofilm carbohydrate did not vary with treatment (Table 2). Extracellular DNA and extracellular protein, when normalized to cell number, were significantly greater for Cr- and Fe+Cr-exposed biofilms (Table 2). Intracellular DNA, expressed on a per-cell basis, was higher for the Fe+Cr treatment than for the Cr treatment (P = 0.02), but other differences were not significant (Table 2). Extracellular carbohydrate appeared to be greater for the Fe+Cr and Cr treatments (Table 2) than for the control and Fe treatments, but differences based on phenol-sulfuric acid assay data were not statistically significant. However, the quantities of EPS sugars determined by GC-MS, when converted to glucose-C equivalents (Table 3), were very similar to the amounts based on the phenol sulfuric acid method (Table 2), and by GC-MS, EPS sugars were significantly more abundant in Cr-exposed biofilms.

When normalized to iDNA, intracellular and extracellular macromolecules followed the same trend across treatments (not shown), i.e., that intracellular protein and carbohydrates were highest for the control treatment and lowest for the Cr treatment. Conversely, extracellular DNA, protein, and carbohydrates were highest for the Cr treatment and lowest for the control treatment.

**EPS sugar composition.** Nine EPS sugars were quantified by GC-MS. The seven commonly detected sugars and their amounts for each treatment are shown in Table 3, except for N-acetylgalactosamine and glucuronic acid, which were only present in the Fe treatment at very low levels. For all sugars except xylose, the Fe+Cr and Cr treatments yielded the highest amounts on a per-cell basis, and except for glucose the amounts were similar across these two treatments. The Fe and control treatments yielded similar masses of sugars and only differed in EPS ribose abundances. Ribose and xylose abundances appeared invariant across treatments (Table 3).

Each sugar in Table 3 was converted to a glucose-equivalent mass as per equation 1. The total glucose equivalents by GC-MS of derivatized EPS were similar and were highest for the Fe+Cr and Cr treatments (Table 3). For all treatments, total glucose equivalents by GC-MS (Table 3) were quantitatively similar to glucose equivalents measured for EPS using the phenol sulfuric acid method (Table 2).

**Metal distribution in biofilms.** By ICP-AES of the EPS and cellular fractions of the biofilms, the Fe+Cr and Cr treatments accumulated a total of 0.16 ± 0.009 μg and 0.11 ± 0.003 μg of Cr per biofilm, respectively. Fe accumulation in the Fe+Cr and Fe treatments was 0.080 ± 0.02 μg and 0.040 ± 0.002 μg per biofilm, respectively. The total Cr content relative to iDNA did not vary between the Cr and Fe+Cr treatments and averaged 0.02 ± 0.003 μg/μg. Similarly, the total Cr concentration in the biofilm did not vary with treatment (P = 0.11) and averaged 0.47 ± 0.19 μg/mm². The Cr content in the biofilms was approximately equally distributed between the cells and EPS. The total Fe content relative to iDNA did not vary across any of the four treatments and averaged 0.012 ± 0.008 μg/μg. Similarly, the total Fe concentration in the biofilm did not vary with

### TABLE 2. Biofilm macromolecular composition normalized to biofilm cell count

<table>
<thead>
<tr>
<th>Treatment</th>
<th>eDNA</th>
<th>iDNA × 10⁵</th>
<th>eProtein</th>
<th>iProtein</th>
<th>eCarbohydrate</th>
<th>iCarbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.014 ± 0.0046⁶ a</td>
<td>4.30 ± 0.82 c</td>
<td>11 ± 2.9 f</td>
<td>15 ± 2.3 h</td>
<td>5.8 ± 0.69 j</td>
<td>6.9 ± 1.71</td>
</tr>
<tr>
<td>Fe</td>
<td>0.013 ± 0.0024⁴ a</td>
<td>9.29 ± 0.45 d</td>
<td>9.3 ± 2.4 f</td>
<td>9.3 ± 0.58 h</td>
<td>3.8 ± 1.8 k</td>
<td>3.7 ± 0.58 i</td>
</tr>
<tr>
<td>Fe+Cr</td>
<td>0.15 ± 0.05⁵ b</td>
<td>4.30 ± 0.13 c, c</td>
<td>71 ± 17 g</td>
<td>18 ± 3.6 h</td>
<td>19 ± 0.8 j, k</td>
<td>4.8 ± 1.31</td>
</tr>
<tr>
<td>Cr</td>
<td>0.071 ± 0.02⁶ b</td>
<td>6.80 ± 0.87 e</td>
<td>58 ± 10 g</td>
<td>6.8 ± 0.58 i</td>
<td>13 ± 3.3 j</td>
<td>1.6 ± 0.58 m</td>
</tr>
</tbody>
</table>

⁶ The prefix “e” denotes the extracellular fraction; “i” as a prefix is for the intracellular fraction. Like letters in each column denote differences that are not significant (t test, P > 0.05).

### TABLE 3. Glycosyl residue content of EPS normalized to biofilm cell count

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Control</th>
<th>Fe</th>
<th>Fe+Cr</th>
<th>Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>0.20 ± 0.036 a</td>
<td>0.16 ± 0.060 a</td>
<td>0.77 ± 0.21 b</td>
<td>0.29 ± 0.031 a, b</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.58 ± 0.32 c</td>
<td>2.27 ± 1.57 c</td>
<td>8.17 ± 1.96 d</td>
<td>6.55 ± 1.00 d</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>0.024 ± 0.0043 e</td>
<td>0.057 ± 0.031 e, g</td>
<td>0.35 ± 0.11 f</td>
<td>0.12 ± 0.027 f, g</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.42 ± 0.078 h</td>
<td>0.49 ± 0.14 h</td>
<td>2.28 ± 0.42 i</td>
<td>1.38 ± 0.25 i</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.90 ± 0.19 j</td>
<td>0.91 ± 0.27 j</td>
<td>5.08 ± 0.74 k</td>
<td>3.56 ± 0.361</td>
</tr>
<tr>
<td>Ribose</td>
<td>ND⁹</td>
<td>0.093 ± 0.047 m</td>
<td>1.68 ± 1.06 m</td>
<td>0.19 ± 0.11 m</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.0056 ± 0.0011 n</td>
<td>0.0098 ± 0.0046 n</td>
<td>0.018 ± 0.014 n</td>
<td>0.0072 ± 0.0038 n</td>
</tr>
<tr>
<td>Total glucose equivalents</td>
<td>3.23 ± 0.65 o</td>
<td>4.10 ± 2.16 o</td>
<td>18.94 ± 4.60 p</td>
<td>11.88 ± 1.82 p</td>
</tr>
</tbody>
</table>

⁹ Like letters in each row indicate differences that are not significant (t test, P > 0.05).

Calculated as per Materials and Methods.

ND, not detected.
treatment ($P > 0.07$) and averaged 0.11 ± 0.04 μg/mm³. However, in all treatments, Fe was associated only with the cells.

Based on the difference between Cr initially adsorbed to the hematite and the final Cr concentration in the Fe+Cr-treated biofilm, approximately 1% of the Cr on the Cr-treated hematite was mobilized into the biofilm. However, little of this mobilization into biofilm, if any, can be attributed to cellular activity, since the mineral medium readily liberated Cr from the hematite (Fig. 3). Also, using the desorption isotherm in Fig. 3 and assuming conservation of mass, calculations showed that approximately 0.20 mg of Cr would have been mobilized into the solid mineral medium by simple (abiotic) desorption and diffusion alone. This would have resulted in an equilibrium concentration of Cr in the solid medium associated with the Cr-coated hematite of 0.05 mM, which was equivalent to the Cr concentration in the solid medium for the Cr-only treatment.

**Cr oxidation state.** Cr oxidation state in the biofilm and biofilm components was assessed by XANES. The spectra for Cr standards show the expected shift of the main absorption edge to higher energies with increasing oxidation state (Fig. 4). In addition, the spectra for Cr(VI) are characterized by a very intense $1s\rightarrow3d$ pre-edge transition at ~5.993 eV, which is almost insignificant in Cr(III). These differences in XANES spectra allow for the determination of the oxidation states of Cr in the samples.

Figure 5 shows the spectra obtained for biofilms produced under the four growth conditions. Biofilms where Cr was not added did not show the presence of any Cr in the sample. The Cr-added and Fe+Cr biofilms both showed that all of the Cr(VI) added to the biofilms had been reduced to Cr(III). If Cr(VI) was present in the samples, it was below the detection limits, i.e., ca. 3% of the total Cr. Samples of separated cells and EPS also showed that all of the Cr in the specific portions of the biofilm had been reduced to Cr(III) (data not shown).

Cr(VI) was added to a control biofilm (2 mM) before XANES measurement to examine the ability of the biofilm to rapidly reduce Cr. Measurements after 10 and 20 min of contact (Fig. 6) show that the reduction of Cr(VI) to Cr(III) is very rapid and is nearly complete after 20 min for these biofilms when not previously exposed to Cr. When control biofilms were exposed briefly to 50 mM Cr(VI), there was no change in the spectra,
suggesting that exposure to the X-ray beam did not cause artifactual reduction of Cr(III) (Fig. 6).

Cr desorption from biofilm EPS. To study desorption of Cr from EPS, biofilms cultured with Cr only were harvested, the EPS dialyzed against deionized water, and the Cr content was measured in the dialyzed EPS. As per Methods, we treated one EPS sample with DNase I, because we hypothesized that eDNA was sorbing Cr(III) and that breakdown of DNA would release sorbed Cr. Three controls were studied for comparison: one of undialyzed EPS, another of dialyzed but untreated (no DNase) EPS, and another, strictly abiotic, control to verify that Cr would readily pass the dialysis membrane. Cr(III) readily passed through the dialysis membrane in that 99.6% of the chromium in Cr-salt samples (no EPS) was removed during dialysis. The total chromium content of the untreated (dialyzed and undialyzed) EPS samples did not differ significantly (t-test, \( P = 0.22 \)) and averaged 0.08 ± 0.05 \( \mu g/ml \), suggesting that Cr did not desorb from the EPS during routine dialysis. However, for the EPS samples treated with DNase I, chromium was not detected in the EPS sample following dialysis, which demonstrated that Cr was sorbed to eDNA in the EPS.

**DISCUSSION**

While Cr(VI) is known to reduce biotically to Cr(III) in the vadose zone (42), the biotic factors controlling the fate of reduced Cr in the vadose zone are not well understood. In this study, unsaturated *P. putida* mt-2 biofilms completely reduced Cr(VI) to Cr(III) and concentrated Cr by nearly 180-fold relative to their external environment. Not surprisingly, Cr(III) was associated with both the cells and EPS. However, surprisingly, eDNA and extracellular Cr(III) were found to be sorbed to one another. Sorption of Cr(III) to eDNA, the latter of which is now known to be common in unsaturated biofilms (54, 55), accounted for high amounts of each in the extracellular matrix of these biofilms and may be a newly identified mode for biotic Cr(III) stabilization in the vadose zone.

Many bacteria can reduce chromate under aerobic and anaerobic conditions (8) where the responsible enzymes are constitutive chromate reductases (30, 32, 44). Accumulation of Cr at rates of 40.7 mg/liter (0.78 mM) total Cr per day in a mixed-species biofilm containing *Pseudomonas* spp. has been reported (11). Rapid chromate reduction to Cr(III) has been observed for soluble chromate reductases isolated from *P. putida* MK1 (44), *P. putida* PRS2000 (30), *Pseudomonas ambiguа* (28), *Arthrobacter* spp., and *Bacillus* spp. (7, 40). Some of these reductases are NADH or NADPH dependent (7, 28, 30, 44), and some, for example, from a soil pseudomonad (39), are not. For the latter isolate, the reductase originated in the cytoplasm, left cells by lysis or secretion, and reduced Cr(VI) extracellularly, leaving Cr deposits on the cell envelope and bound to EPS and in solution (39). Cr(III) was both cellular and extracellular in our study, and thus, it is possible that cell lysis released constitutive reductases that catalyzed the extracellular reduction of Cr(VI) to Cr(III). Regardless of where Cr(VI) occurred, our previously frozen, control biofilms rapidly reduced Cr(VI) to Cr(III) (Fig. 6), which supports that the process was constitutive.

The native ability to reduce Cr(VI) to Cr(III) is important given that Cr(VI) is toxic to bacteria. In the present study, the Cr(VI) concentration in the Cr-only treatment (0.05 mM as K₂Cr₂O₇ or 0.1 mM as Cr) and the amount of potassium dichromate-coated hematein in the Fe+Cr treatment were both selected to avoid severe toxicity based on prior reports. For example, toxicity thresholds reported for one planktonic *Pseudomonas* sp. were 4.3 mg liter⁻¹ (0.083 mM) in succinate and 8.8 mg liter⁻¹ (0.17 mM) in Luria-Bertani broth (33). The toxicity threshold for *P. aeruginosa* A2Chr was between 0.1 mg/liter (0.19 mM) and 40 mg/liter (0.77 mM) (22, 23) and for another *Pseudomonas* sp. was 10.4 mg/liter (0.2 mM) Cr(VI) (64). These studies were for planktonic cultures, but toxicity thresholds were similar for biofilm-cultivated and planktonic cells when investigated across several bacterial species, including *P. aeruginosa* (25). In our study, cells were obviously stressed, as indicated by the lower yield, lower protein and carbohydrate levels per cell, and increased extracellular macromolecules in biofilms exposed to Cr. Many different environmental stresses have been previously reported to effect an increased production of extracellular carbohydrates in biofilms. Desiccation resulted in increased polysaccharide production by a *Pseudomonas* sp. (47). Trivalent chromium resulted in a nearly 82% increase in extracellular carbohydrate in sulfate-reducing bacterial biofilms (18). Mix-species sulfate-reducing bacterial biofilms also exhibited an increase in extracellular carbohydrates when exposed to cadmium, possibly as a result of metal binding polymer production (71). *P. putida* biofilms exposed to toluene showed a significant increase in extracellular carbohydrate, and the composition shifted towards increasing carboxylic groups (48). We also observed changes in the carbohydrate composition of *P. putida* biofilms exposed to Cr. All of the sugars shown in Table 3 were present in significantly greater amounts in the Cr and Fe+Cr treatments than in the control treatment. The greatest increases were for glucose, N-acetyl-galactosamine, mannos, and rhamnose. As for *P. aeruginosa* (55), glucose and rhamnose were the predominant biofilm saccharides. For *P. ambiguа*, glucose protected the chromate reductase enzyme from inactivation (28), which may be a role for EPS glucose in this study. The EPS glycosyl changes could also facilitate cation binding, as was proposed by Schmitt et al. (48), and could lead to the retention of soluble Cr(III) as an organo-complex (46). On the other hand, Cr(III) may bind to many macromolecules besides polysaccharides (46). Our work suggests a particularly important role for eDNA.

EPS was clearly enriched in eDNA relative to intracellular concentrations, which suggests that although DNA was liberated into the EPS, it was also stabilized. In this study, eDNA relative to cellular DNA was high for all treatments but exception only so for biofilms exposed to Cr (e.g., 10-fold higher in the Cr-only treatment). Cellular DNA was conserved across treatments and matched expected femptogram levels for laboratory and environmental bacteria (45), suggesting that the eDNA levels reported here were not methodological artifacts. DNA in bacterial EPS has been reported previously for saturated (57) and unsaturated (54, 55) biofilms with a suggested release mechanism of cell lysis. DNA can also be transported outside the cell by vesicles and is necessary for early *P. aeruginosa* biofilm growth (70). However, we believe that enhanced EPS macromolecules in our study were probably due to accelerated cell lysis, as reported previously for an anaerobic consortium exposed to chromium (3). As evidence, we observed that the
proportions of intracellular proteins and carbohydrates were preserved extracellularly.

The extracellular accumulation of both DNA and Cr(III) implies an association between these two molecules in the presence of intracellular proteins and carbohydrates were performed the GC-MS quantification of glycosyl residues.

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