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ESTIMATION OF CELLULAR AND EXTRACELLULAR CARBON CONTENTS IN SULFATE-REDUCING BACTERIA BIOFILMS BY LIPOPOLYSACCHARIDE ASSAY AND EPIFLUORESCENCE MICROSCOPIC TECHNIQUE

S. OKABE*, P. H. NIELSEN†, W. L. JONES and W. G. CHARACKLIS

The Center for Biofilm Engineering, Cobleigh Hall 409, Montana State University, Bozeman, MT 59717, U.S.A.

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Abstract—Measurement of cellular and extracellular carbon contents of sulfate-reducing bacteria (SRB) is essential and important in studies of the role of SRB in corrosion and biofouling. An epifluorescence (EPI) microscopic technique and a lipopolysaccharide (LPS) assay were used to quantify cellular and extracellular carbon contents in *Desulfovibrio desulfuricans* biofilms. The average contents of lipopolysaccharide (LPS) and cellular carbon were 7.3 ± 2.8 (fg LPS) cell^{-1} and 39.9 ± 9.9 (fg cellular-C) cell^{-1}, respectively, in a *D. desulfuricans* chemostat culture. A ratio of cellular carbon content to LPS content was 6.5 ± 2.8, and was used to estimate cellular carbon contents in a *D. desulfuricans* biofilm. The LPS and EPI methods gave comparable results for suspended samples, but not for biofilm samples.

Key words—*Desulfovibrio desulfuricans*, cellular carbon, biofilms, extracellular polymeric substance (EPS), lipopolysaccharide, limulus amebocyte lysate, epifluorescence microscopic technique (EPI).

INTRODUCTION

Measurement of cellular biomass and extracellular products of sulfate-reducing bacteria (SRB) is essential and important in studies of the role of SRB in corrosion and biofouling. Biofilms containing SRB are responsible for metal and concrete corrosion (Mori *et al.*, 1991; Lee *et al.*, 1992) and H_2S production in oil producing industries (Sanders and Hamilton, 1985; Frazer and Bolling, 1991) and wastewater treatment systems (Nielsen, 1987; Nielsen *et al.*, 1988). Separation of biofilms into two components, cellular mass and extracellular polymeric substance (EPS) is essential to successfully determine microbial activity and cell yield (Bakke *et al.*, 1984; Okabe and Characklis, 1992; Okabe *et al.*, 1992), especially in biofilm systems containing a large amount of EPS.

In general, SRB biomass is frequently measured as dry weight (suspended solids), total organic carbon (TOC), and total cellular protein contents. However, measurements of SRB biomass using drying/gravimetric methods and by measuring turbidity are frequently subject to errors due to iron sulfide production (Reis *et al.*, 1991; Okabe *et al.*, 1992). Moreover, cellular protein measurement for SRB biomass frequently proved unreliable due to high concentrations of iron sulfide (Reis *et al.*, 1991) and the presence of an extensive amount of extracellular protein in biofilms. Therefore, a reliable and convenient alternative method needs to be developed.

The epifluorescence (EPI) microscopic technique developed by Hobbie *et al.* (1977) has been used to enumerate SRB cell numbers and to estimate cellular carbon and EPS carbon contents (Okabe and Characklis, 1992; Okabe *et al.*, 1992). It has been reported that the EPI technique requiring conversion of cell volume to cellular carbon content is frequently subject to errors due to cell size fluctuation (Watson *et al.*, 1977). Since the EPI technique has not been applied to SRB biofilm samples, its applicability to SRB biofilm samples needs to be investigated. The purpose of this manuscript was to investigate the applicability of the EPI technique in measuring SRB cellular and EPS carbon contents and to compare with a lipopolysaccharide (LPS) assay technique, which has been also used for the measurement of bacterial numbers and biomass (Watson *et al.*, 1977).

DETERMINATION OF CELLULAR-C/LPS RATIO

To estimate cellular carbon content from LPS content, a ratio of cellular carbon and LPS contents of *Desulfovibrio desulfuricans* (ATCC 5575) cells was determined in a chemostat culture at various dilution rates (D) (Table 1). *D. desulfuricans* was grown in a modified Postgate medium *G* (Postgate, 1984)

*Present address: Department of Civil and Environmental Engineering, Miyazaki University, Miyazaki, 889-21, Japan.

†Present address: Environmental Engineering Laboratory, Aalborg University, DK-9000 Aalborg, Denmark.

containing 1.24 g l^{-1} of Na–lactate and 1.0 g l^{-1} of Na_2SO_4. Selenite and sulfide solutions were omitted. Temperature was maintained at 35°C and pH at 7.0. The details of chemostat set-up protocol and operating procedure have been described elsewhere previously (Okabe and Characklis, 1992). Previous chemostat data (Okabe and Characklis, 1992) and visualization of EPS production by EPS staining (Allison and Sutherland, 1984) indicated that *D. desulfuricans* grown under such an experimental condition in the chemostat did not produce significant EPS. Thus, the bacterial cellular carbon was determined as the difference between the effluent total organic carbon (TOC) and soluble organic carbon (SOC). The samples (20 ml) for TOC analysis were homogenized by a homogenizer (Tekmar, Cincinnati, OH) for 1 min. The samples (45 ml) for SOC analysis were prepared by centrifugation at 20,000 ***g***, 4°C for 20 min using stainless steel centrifuge tubes. The supernatant was used for the SOC determination. All samples for TOC and SOC were analyzed using an ultraviolet-promoted persulfate oxidation followed by infrared detection (Dohrmann DC-80 total organic carbon analyzer). Lipopolysaccharide was determined spectrophotometrically. Limulus amebocyte lysate for spectrophotometric method (Associates of Cape Cod, Inc., code No. 100-K5) was obtained in freeze-dried form (5 ml/vials) and was reconstituted immediately before use by the addition of 5 ml of pyrogen-free water and stored on ice during use. *Escherichia coli* endotoxin (Associates of Cape Cod, Inc., code No. 800-1) was used as the standard. More details of analytical procedures have been described elsewhere previously (Sullivan *et al.*, 1976).

Average cell number and cell volume were determined using the EPI technique. Effluent samples were homogenized with a tissue homogenizer and preserved in 2% (final concentration) formaldehyde solution at 4°C until analysis. The samples were incubated for 10–20 min with 0.01% (final concentration) acridine orange and filtered through a 25 mm, 0.2 μm pore size black polycarbonate membrane filters (Poretics Corporation, Livermore, CA). The filter was then examined with an Olympus AH-2 microscope and image analysis system (Videometric 150, American Innovations, San Diego, CA) to determine the cell size and numbers. More details of this method have been described elsewhere previously (Hobbie *et al.*, 1977). Cell numbers were reported as the mean of 20 frame measurements along the filter. Cell sizes were reported as the mean of more than 100 direct measurements per sample. Average cell volumes were estimated using the average cell sizes. Cells were assumed to be represented as short cylinders with a half-sphere on each end, the half-sphere having a radius equal to half of the cell width. The cell width was relatively constant ($w = 0.5\ \mu$m) in this study. To convert cell volume to cellular carbon, the following factors were used: wet specific gravity (1.07×10^{-6} g wet cell $(cm^3)^{-1}$) (Doetsch and Cook, 1973), dry cell mass to wet cell mass ratio (0.22 g dry cell [g wet cell]$^{-1}$) (Luria, 1960), and cellular carbon content (0.5 g cellular carbon [g dry cell]$^{-1}$) (Luria, 1960). As shown in Table 1, an average of the experimentally measured cellular carbon content was 39.9 ± 9.9 fg cell^{-1}, reasonably agreed to the estimated cellular carbon content from the cell size of 29.4 ± 4.1 fg cell^{-1}, indicating that the measured cellular carbon content was reliable. An average cellular-C/LPS ratio was 6.5 ± 2.8 and agreed with the cellular-C/LPS ratio of 6.3 ± 2.0 for *E. coli* obtained by Watson *et al.* (1977). The cellular-C/LPS ratio decreased with decreasing the cell volume, suggesting that the cellular-C/LPS ratio is related closely to the cell surface and volume ratio (A/V). The LPS content of *D. desulfuricans* cells (7.3 ± 2.8 fg cell^{-1}) was low as compared with the LPS content/cell range of 10–40 fg cell^{-1} for other gram-negative bacteria (Sullivan *et al.*, 1976; Watson *et al.*, 1977; Hurley and Tosolini, 1992; Mattsby-Baltzer *et al.*, 1991). The reason for low LPS content for *D. desulfuricans* is that cell volume of *D. desulfuricans* is smaller than *E. coli*. An average cell volume of *D. desulfuricans* determined in this study was 0.26 μm^3, whereas *E. coli* typically has a larger average cell volume of around 1.6 μm^3 (Watson *et al.*, 1977). Dependence of cellular carbon and LPS contents per cell on specific growth rate could not be evaluated in this study due to fluctuation of experimental data. A possible reason for the fluctuation in cellular carbon content could be that separation of cells from growth medium by the centrifugation was not completely performed. The fluctuation in LPS content could be attributed to that distinction between free LPS and bound LPS was not made in this study. Because bound LPS is more closely correlated with cell number than total LPS

Table 1. LPS and cellular carbon contents in *D. desulfuricans* cells from chemostat cultures at various dilution rates (AVG ± SD)

D (h^{-1})	Cell number (cells × 10^8 ml^{-1})	Cellular-C (fg cell^{-1})	LPS (fg cell^{-1})	Cellular-C/LPS (fg fg^{-1})	Average cell vol. (μm^3)	Estimated cellular-C by EPI (fg cell^{-1})
0.05	1.06 ± 0.05	38.5	7.0	5.5	0.271	31.9
0.08	1.78 ± 0.09	26.7	2.6	10.3	0.283	33.3
0.10	1.04 ± 0.12	50.7	5.6	9.1	0.256	30.1
0.12	1.00 ± 0.08	50.7	10.4	4.9	0.236	27.8
0.12	1.34 ± 0.18	34.7	9.1	3.8	0.213	25.1
0.12	1.55 ± 0.16	28.1	10.9	2.6	0.193	22.7
0.24	0.88 ± 0.04	50.1	5.5	9.1	0.295	34.7
Average		39.9 ± 9.9	7.3 ± 2.8	6.5 ± 2.8	0.259 ± 0.035	29.4 ± 4.1

during logarithmic phase (Watson *et al.*, 1977). The LPS assay also associates a relatively high analytical error, possibly because of the greater number of steps that are necessary.

DETERMINATION OF CELLULAR AND EPS CARBON CONCENTRATIONS IN SRB BIOFILMS

To investigate the applicability of the EPI and LPS methods to SRB biofilm samples, a monopopulation of *D. desulfuricans* biofilm was grown in a continuous-flow, annular biofilm reactor (RotoTorque^{tm}, ERC, Montana State University, Bozeman, MT) (Bakke *et al.*, 1984). Twelve removable polycarbonate slides installed on the inside wall of the outer cylinder were used to sample the biofilm. Anaerobic conditions were maintained by a continuous nitrogen gas purge. The pH was maintained at 7.0, and temperature at 35°C. A typical biofilm thickness progression is presented in Fig. 1. Biofilm thickness was determined with an Olympus BH-2 microscope using the method of Bakke and Olsson (1986). Biofilm thickness was reported as the mean of 8 measurements along a slide. The biofilm thickness gradually increased to about 60 μm at 15 h and then decreased due to a biofilm sloughing. The standard deviation increased with increasing the biofilm thickness, exhibiting an increase in substantial heterogeneity in thickness. RotoTorque^{tm} biofilm coupons were removed aseptically from the reactor and the biofilm was rinsed twice with deionized water, scraped into 50 ml of deionized water from a known surface area, and homogenized using the tissue homogenizer. The homogenized biofilm samples were analyzed for TOC, SOC, LPS, cell size, and cell number as described above. Areal cellular carbon density was determined multiplying the measured areal LPS density by the experimentally determined cellular-C/LPS ratio (6.5 ± 2.8) and multiplying the measured areal cell density by the conversion factors for cell volume to cellular carbon as described above [Fig. 2(a) and (b)]. Areal EPS carbon density was determined

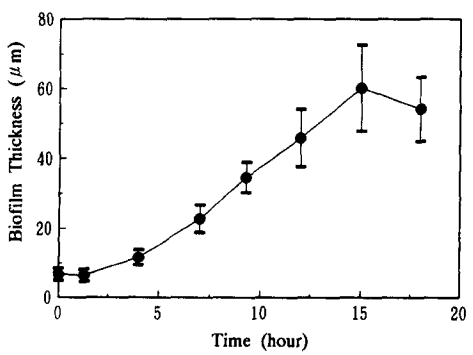

Fig. 1. A typical progression of *D. desulfuricans* biofilm accumulation. Error bars represent the standard deviations of measurements ($n = 8$).

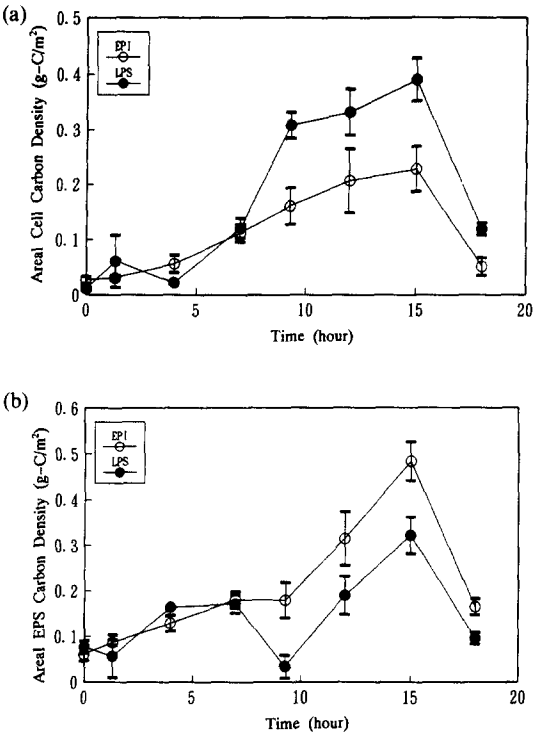

Fig. 2. Typical progression curves of (a) biofilm cellular carbon and (b) biofilm EPS carbon areal densities with time determined by the LPS assay and the EPI method. Error bars represent the standard deviations of measurements ($n = 2$).

subtracting the areal cellular carbon density from the total biomass carbon (TOC–SOC) density.

There was no significant difference between the values of cellular carbon and EPS carbon densities measured by both methods in the initial biofilm accumulation phase. However, as biofilm accumulated thicker (after 7 h), the LPS data did not agree to the EPI data [Fig. 2(a) and (b)]. The areal cellular carbon density measured by the LPS method was higher than the EPI data. This is partly because since bacterial cells constantly release LPS (Sullivan *et al.*, 1976), the released LPS accumulated in the biofilm, leading to overestimation of cellular carbon content. It could also be possible that the cellular-C/LPS ratio of *D. desulfuricans* in the biofilm differs from one in suspended growth, even though the average cell size of biofilm cells and its standard deviation were relatively constant over the experimental course and were close to the values of suspended cells.

PRACTICAL APPLICATION AND CONCLUSION

Standard deviations of both data were comparable, indicating that comparable precision was obtained by the two techniques. Sources of error in the two methods are not the same, however, and there may be experimental advantages on one technique over the other, depending on the sample type. The result of this study indicated that the EPI and LPS techniques

gave a comparable result for the suspended samples, but not for biofilm samples.

The minimum detectable concentration of LPS is 1–5 pg ml^{-1} which corresponds to *D. desulfuricans* concentration of 1.4–6.8 × 10^2 cells ml^{-1}. The detection limit of the epifluorescence direct count varies depending on sample volumes. Thus, relatively low cellular carbon concentrations can also be determined accurately using both techniques. Although the minimum detectable concentration of TOC is 0.5–1.0 mg-C l^{-1} which corresponds to bacterial concentration on the order of 1.3–2.5 × 10^7 cells ml^{-1}, bacterial samples can be concentrated by centrifugation and filtration to obtain the necessary biomass concentration. Thus, the separation of total biomass into cellular mass and EPS can be performed at low biomass concentrations.

An issue of great interest is the applicability of these methods to undefined mixed population samples, in which considerable variations of cell size and cellular-C/LPS ratio within a bacterial population are expected. This problem could be solved analyzing cell size distributions within a bacterial population and converting cell numbers to cellular carbon concentrations using the cell size distribution. Also if a correlation between the cellular-C/LPS ratio and the cell surface and volume ratio is determined, the cellular carbon concentration could be determined by incorporating the determination of cell size distribution by the EPI into the LPS method. For application to biofilm samples, these methods must be used cautiously because of accumulation of released LPS. However, this problem could be overcome separating released LPS from total LPS using a centrifuge at 8000 *g* for 10 min (Watson *et al.*, 1977). The supernatant must be assayed for released (free) LPS. Thus, bound LPS can be determined subtracting the free LPS from the total LPS. It is apparent that further investigation is needed to develop a simple and reliable means to determine the cellular and extracellular carbon contents in mixed population biofilms.

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