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Quantification of *Dehalospirillum multivorans* in Mixed-Culture Biofilms with an Enzyme-Linked Immunosorbent Assay

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A fast, highly selective and sensitive method to quantify specific biomasses in mixed-culture biofilms is described. It consists of detachment of a biofilm from its support material, resolution of the detached biofilm flocs in order to separate the enclosed cells and antigens, and quantification of specific biomass by an enzyme-linked immunosorbent assay.

Quantification of microbial biomass is an important factor for optimization and performance of biotechnological processes. Because of the lack of suitable methods, the degradation of organic substances in biological treatment plants is usually related to summary parameters, like amounts of volatile suspended solids or dry weight. The application of rates, calculated on the basis of these parameters, to other systems (e.g., varying waste water composition) is questionable, since these parameters grossly misrepresent the microbial populations responsible for metabolic processes (14). In order to obtain turnover rates of general applicability, methods are needed to determine specific biomasses in bioreactors for the purification of water (e.g., wastewater and contaminated groundwater). It is clearly desirable to extend these methods to biofilms, which play a central role in the degradation of organic substances in biotechnological processes as well as in natural ecosystems (2).

In this paper a technique is presented that has been developed to quantify the biomasses of the anaerobic bacterium *Dehalospirillum multivorans* in mixed-culture biofilms. This bacterium is a particularly interesting example because of its potential applicability to the purification of groundwater contaminated with tetrachloroethene, since it is able to dechlorinate tetrachloroethene by its energy metabolism via trichloroethene to *cis*-1,2-dichloroethene at high turnover rates (7, 8, 13). It was isolated from activated sludge and belongs to the epsilon subdivision of proteobacteria (13). In order to obtain a model biofilm, an anaerobic fluidized-bed reactor was inoculated with *D. multivorans* and operated under nonsterile conditions.

Biofilms are special environments where microorganisms are firmly attached to surfaces and to one another by exopolymeric substances (EPS). Classical techniques to enumerate specific bacteria, e.g., by counting CFU or by using the most probable number technique, are certainly not suited to produce the accuracy needed to determine kinetic parameters for reactor designs. Furthermore, EPS preclude the use of direct enumeration techniques (e.g., immunofluorescence microscopy) since they prevent antibodies from reacting with the enclosed bacteria (16). To separate the biofilm into single cells as needed by immunofluorescence microscopy and to remove the EPS, mechanical forces (e.g., ultrasonication) and/or chemical treatment are needed. During this treatment a high percentage of the cells is destroyed (15). Consequently, the cell number will be greatly underestimated in a mixed-culture biofilm by microscopical methods.

Thus, clear difficulties in quantification of specific bacteria in biofilms are the complete separation of the attached bacteria from the support material and their enumeration. Recently, enzyme-linked immunosorbent assays (ELISAs) have proven to be powerful tools to quantify specific biomasses in several systems (3, 6, 9, 17, 21). However, their application to multilayer aquatic biofilms has until now not been possible, since it has to be guaranteed that the antibodies can react with all the antigens in order to have a correct quantification. This paper presents a balanced combination of mechanical and chemical treatments that circumvent these difficulties, so that for the first time a quantitative determination of specific biomass by ELISA is possible with aquatic biofilms.

To obtain polyclonal antibodies against *D. multivorans*, cell suspensions were grown anaerobically in mineral medium with pyruvate as the only energy and carbon source (7). The cells (10^9 cells per injection) were injected into a New Zealand White rabbit. Following four booster injections at 1-week intervals, blood samples were collected from the marginal ear vein. For application to the ELISA, the antiserum was purified by fast liquid chromatography on a protein A-bead column. An aliquot of the immunoglobulin G fraction was labeled with biotin by coupling with D-biotinylamido-caproic acid-*N*-hydroxysuccimidester (Boehringer, Mannheim, Germany). The immunoassay was performed as an antibody-capture assay according to the following protocol. Microtitration plates (Greiner GmbH, Solingen, Germany) were coated with purified anti-*D. multivorans* immunoglobulin G at a dilution of 1:1,000 (vol/vol) and incubated for 16 h at 4°C. Unspecific binding sites were blocked by adding 0.1% bovine serum albumin (BSA; Sigma GmbH, Deisenhofen, Germany) in phosphate-buffered saline (PBS). The plates were stored at −20°C until use. Biofilm samples or cell suspensions of *D. multivorans* in PBS (200 μl per well) were added to an antibody-coated plate and left for 16 h at 4°C. Subsequently, the plate was washed in PBS and the biotinylated anti-*D. multivorans* immunoglobulin G antibody (200 μl per well), diluted 1:1,000 in PBS with 0.1% BSA, was added. After a reaction time of 100 min at 37°C, the plate was washed and the assay was completed by incubating with Extr-Avidin-peroxidase (Sigma GmbH; 200 μl per well) diluted 1:1,000 in PBS with 0.1% BSA. After 45 min at room temperature, the plate was washed with PBS and the substrate solutions (urea hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine) for the peroxidase reaction were added and left for 15 min at room temperature in the dark. The

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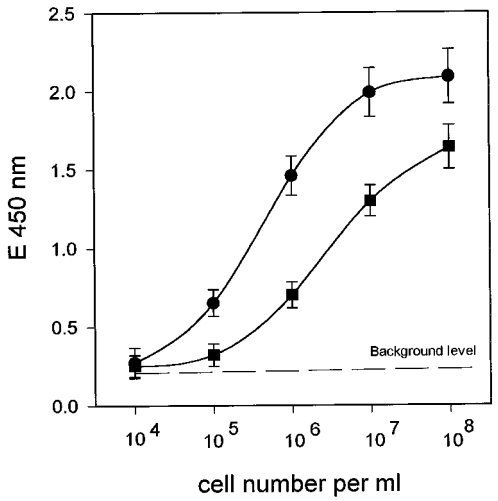

FIG. 1. Influence of the nutrient conditions on the detection limit of the ELISA for *D. multivorans*. ELISA standard curves for *D. multivorans* grown in mineral salt medium with pyruvate, fumarate, and yeast extract (●) and in mineral salt medium with formate, tetrachloroethane, and acetate (■) are presented.

reaction was stopped by adding 50 μl of sulfuric acid (2 N), and the optical densities of the wells were read at 450 nm on a plate reader (MR 5000; Dynatech, Denkendorf, Germany).

In order to test the specificity of the polyclonal antiserum, the following anaerobic bacteria were examined by ELISA. *Campylobacter* sp. DSM 6222 and *Wolinella succinogenes* DSM 1740 were chosen, since they are related to *D. multivorans* according to 16S rRNA analysis (13). *Desulfovibrio* sp. DSM 2480, a representative of sulfate-reducing bacteria, was tested as were *Peptostreptococcus* sp. and *Clostridium formicoaceticum*, which represent the ecologically important group of the homoacetogenic bacteria, and finally the methylchloride-degrading strain MC. Like *D. multivorans*, strain MC was isolated from wastewater (18). *Peptostreptococcus* sp., *Clostridium formicoaceticum*, and strain MC were obtained from G. Diekert, Institute of Microbiology, University of Stuttgart. In addition, we tested an anaerobic mixed culture, which was prepared by incubating sewage sludge in anaerobic medium (13) with additions of glucose, pyruvate, and yeast extract. None of the tested pure cultures nor the mixed anaerobic culture cross-reacted in the ELISA, showing that our ELISA is highly specific for *D. multivorans*.

For quantification of *D. multivorans* in mixed culture, ELISA standard curves were obtained with suspended cells of the pure culture of *D. multivorans*. The cell number of the standard cell suspension was determined by fluorescence microscopy (4). In the ELISA, the optical densities of these standard cell suspensions varied from 0.25 as the background value to about 2.4 over a range of 10^4 to 10^8 cells per ml. The detection limit of the assay, calculated by adding 2 standard deviations to the zero value, varied depending on the nutrient conditions as follows. Cells grown in a mineral salt medium with pyruvate (40 mM), fumarate (40 mM), and yeast extract (0.1%) could be detected down to 10^5 cells per ml, whereas *D. multivorans* cells grown in the mineral salt medium with formate (20 mM), tetrachloroethene (10 mM, in hexadecane), and acetate (5 mM) could be detected to 10^6 cells per ml (Fig. 1). By cultivating the standard cells used in the ELISA under the same nutrient conditions used for the biofilm samples, we considered the influence of the nutrient composition. The detection limit of *D. multivorans* was not affected in the presence of large numbers of possibly competing cells, since no influence on the detection of a specific biomass in the ELISA could be observed down to at least 0.01% of specific cells in a mixture with nonspecific cells (data not shown).

In order to quantify specific biomass in a mixed culture biofilm by ELISA, the biofilm was to be detached from the support material and separated into single cells or into antigens. For optimization of this procedure, biofilm from a fluidized-bed reactor (0.8 liter) inoculated with *D. multivorans* and nonspecific bacteria was used. The biofilm was attached to sintered glass beads (diameter, 350 μm; Poraver) and supplied continuously with formate (5 mM), tetrachloroethene (0.15 mM), and acetate (1 mM) for a retention time of 12 h. Biofilm detachment was studied by ultrasonication. Samples from the fluidized bed (approximately 2 g [wet weight]) were suspended in PBS and ultrasonicated (with a Branson [Danbury, Conn.] sonifier; diameter of ultrasonication tip, 1 cm) at different intensities and for different times in a volume of 4 ml (vessel, 20 ml; diameter, 2 cm) under cooling conditions in an ice bath. The efficiency of the biofilm detachment was determined by measuring the protein content of the support material, according to the method of Lowry et al. as noted in the report of Peterson (10), before and after ultrasonication. After an incubation time of 5 × 1 min (40% output), 90% of the biofilm was detached. Increasing the ultrasonication intensity or the incubation time did not increase the detachment efficiency (data not shown).

While removing the biofilm from the glass beads by ultrasonication, the cells were destroyed to a great extent. To study the effect of cell destruction on the ELISA, suspended cells of the pure culture (standard cells) were ultrasonicated under the same conditions described above, by which 90% of the cells were destroyed as determined microscopically. There was no significant effect of cell destruction on the ability of the ELISA to detect specific cells. Identical ELISA readings were obtained when the standard cells were treated with a French press, which disintegrated the cell structure more effectively than ultrasonication, as monitored by phase-contrast microscopy.

According to our observations, ultrasonicated biofilms usually detach in flocs containing numerous single cells. As has been shown with nitrifying biofilms (16), the reaction of the antibodies with the antigens inside these flocs is prevented by the biofilm matrix (EPS). Consequently, the EPS has to be removed in order to quantitatively determine the number of *D. multivorans* organisms in the mixed-culture biofilm. The effects of different chemicals on the separation of the biofilm flocs, as monitored by phase-contrast microscopy, are listed in Table 1. Treatment of the flocs with NaCl, which increased the immunofluorescence count of *Nitrosolobus* sp. in nitrifying biofilms (16), had no effect on the flocs of the anaerobic dechlorinating

TABLE 1. Chemicals tested to disintegrate biofilm flocs detached from support material of a dechlorinating fluidized-bed reactor

Chemical treatment[a]	Microscopic observation (size in μm)	Cell no. per ml by ELISA
Control (no treatment)	Many flocs (50 to 100)	5×10^6
1 M NaCl	Many flocs (50 to 100)	6×10^6
0.1% Tween 80	Single flocs (20 to 50)	2×10^8
5 mM EDTA	No flocs detectable	ND[b]
0.1% Na-polyphosphate	No flocs detectable	8×10^8

[a] Samples were incubated for 1 h at 30°C on a rotary shaker.
[b] ND, not determined.

biofilm. With the detergent Tween 80, the flocs could be resolved to a high degree. Complete disintegration of the flocs, however, was possible with Ca-specific chelants like Na-polyphosphate and EDTA. The optimum concentration of these reagents, resolving the flocs completely, was 5 mM EDTA or 0.1% Na-polyphosphate, respectively, at an incubation time of 1 h on a rotary shaker (Table 1). For further experiments, 0.1% Na-polyphosphate was used to destroy the flocs. At this concentration, no interference of the reagent with the immune reaction occurred. Resolving the biofilm flocs by this method increased the ELISA cell count of *D. multivorans* by 2 orders of magnitude.

The effect of EDTA or polyphosphate is based on the reaction with two-valent cations, especially calcium ions, since bridging with Ca^{2+} ions is one of the mechanisms stabilizing the biofilm matrix (5). Hence, in the case of *D. multivorans*, complete elimination of these bivalent ions from the biofilm clearly helps to destroy the network of the EPS, releasing the enclosed cells or antigens.

By the procedure described above, a cell number of *D. multivorans* of 5×10^{12} cells per g of total protein could be determined in the dechlorinating biofilm of a fluidized-bed reactor supplied continuously with formate as the electron donor, tetrachloroethene as the electron acceptor, and acetate as the carbon source. In order to relate the cell count of specific tetrachloroethene-dechlorinating cells to the total biomass of the biofilm, the cell number of *D. multivorans*, as determined by ELISA, was expressed in terms of protein, assuming that 10^{10} cells are equivalent to 1.5 mg of protein. This value was determined for *D. multivorans* as average protein content during growth. By converting the cell number into the protein content, approximately 75% of the total biomass of the biofilm in the reactor could be assigned to *D. multivorans*. On the basis of these data, a specific rate of about 60 nmol/min/mg of cell protein was determined for the dechlorination of tetrachloroethene to trichloroethene and a rate of 90 nmol/min/mg of cell protein was determined for the dechlorination of trichloroethene to *cis*-1,2-dichloroethene (incubation temperature, 20°C). The dechlorination rate was lower by a factor of 3.5 than that of cell suspensions of the pure culture (incubation temperature, 30°C). This could only partly be due to the different incubation temperatures. Another important difference between the two systems was the age of the specific cells. For cell suspension experiments, bacteria in the logarithmic growth phase were used. In biofilms, on the other hand, the retention time was usually high. Consequently, a high percentage of the biomass was inactive or nonviable and the turnover rate calculated from the total specific biomass was underestimated. In order to determine the real degradation capacity of a specific bacterial group in a mixed-culture biofilm, it would naturally be desirable to differentiate between active and inactive specific biomasses. Unfortunately, no method has been available until now to determine active specific biomasses in biofilms. Even staining of actively respiring microorganisms with tetrazolium salts in combination with immunofluorescence microscopy for detection of specific bacteria (1) cannot be applied to complex biofilms because of the difficulties presented by detaching bacteria, discussed in detail in this paper. These direct microscopical methods are restricted to the enumeration of planctonic bacteria or of monolayer biofilms (12). However, the present method makes important progress compared with the summary parameters used until now to describe the dynamic processes in a mixed-culture biofilm.

In summary, our results show that it is possible to quantify the total biomass of specific organisms responsible for the degradation of organic pollutants in mixed-culture biofilms. Therefore, it is expected that specific turnover rates of general applicability can be determined to describe the process of contaminant detoxification in mixed-culture biofilms under different conditions.

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