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Ciliate contributions to bioaggregation: laboratory assays with axenic cultures of *Tetrahymena thermophila*

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Summary. Protists, mainly ciliates, play several essential roles in biological wastewater treatment, such as the transfer of matter and energy, bacterial predation, and the removal of organic material. Moreover, during the treatment process, the formation of bioaggregates—flocs and biofilms—is essential to obtaining high-quality effluents. In the present study, *Tetrahymena thermophila* was used as a model organism to demonstrate the contribution of ciliates to bioflocculation. Axenic cultures of this species were exposed to chemical and mechanical stimuli that promote bioaggregation. In either case, the secretion of a capsulate mucous material by the ciliates or by particle aggregation was detected. Numerous, small, loosely compacted flocs were observed under shaking conditions and in the presence of latex beads. The composition of the exopolymeric material secreted by ciliates was analyzed by a series of fluorochromes and colorimetric methods, which showed that carbohydrates and nucleic acids were the main components involved in matrix formation and particle adhesion. [**Int Microbiol** 2007; 10(2):91-96]

Key words: *Tetrahymena thermophila* · waste water treatment · flocculation · extracellular polymeric substances (EPS)

Introduction

Bioaggregate formation is one of the most important ecological strategies of microorganisms for the efficient colonization of different natural and artificial habitats. The main step in the development of microbial consortia is the formation of an extracellular matrix, which is produced by the biological activity of the bacteria [15,24], protists, and other organisms that support the structure of the bioaggregates. This matrix consists of extracellular polymeric substances (EPS), mostly polysaccharides and proteins, although small amounts of nucleic acids, amino acids, and lipids have also been described [10,19]. Due to their hydrophobic properties and their charge, bioaggregates allow the accumulation of nutrients, constitute an ecological niche that protects the microbial community against toxic injury, diminish the accessibility of constituent microbial cells to predation, and foster genetic exchange [1,6,12,28].

In artificial environments, such as in biological reactors of wastewater treatment plants (WWTP), microbial communities involved in the elimination of organic matter and other contaminants of the input are established as flocs or biofilms, depending on the design of the reactor. The effluent quality depends on the capacity to remove the bioaggregates—flocs or biofilms—from the purified water, which is returned to natural habitats. In a previous study, we analyzed the structure and composition of exopolymeric substances in flocs and biofilms of rotating biological contactors. These substances consist of proteins, humic-like material, and polysaccharides, which are the main components [18]. Other authors have described the flocs of activated sludge plants as aggregates of cells surrounded by a fibrous matrix of exopolysaccharides [7].

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Together with bacterial populations, protists—mainly ciliates—are the principal functional microbial community that promotes wastewater treatment. They are essential for the transfer of matter and energy through different trophic levels [25], preserve the stability of bacterial populations [5], and remove organic matter, either dissolved [13] or flocculated [22]. In addition, several authors have suggested that protists contribute to the flocculation process by the secretion of polymeric compounds [4,31].

The main objective of the present study was to demonstrate the role of ciliates in biological aggregate formation and the importance of this process in the biological treatment of wastewater. Due to the complexity of the microbial communities that are involved in the biological treatments carried out in WWTP, the first step was to confirm whether ciliates growing in axenic culture, i.e., in the absence of other populations, can undergo bioaggregation. To this end, the ciliate *Tetrahymena thermophil*a was selected as a model organism, because this species, reported in WWTP [9], can be easily grown in stable axenic culture.

Materials and methods

Culture conditions. Axenic cultures of *Tetrahymena thermophila* (CCAP 1630-1M) were maintained in proteose peptone glucose medium (PPG-Cultimed 403939.1210) containing an antibiotic antimycotic solution (Sigma A5955). The absence of bacteria was confirmed by periodic inoculation of samples in tryptone soy agar.

Exocytosis experiments. Alcian blue (Sigma, CAS 75881-23-1) was used as an inducer of cell exocytosis, following the protocol described by Turkewitz and Kelly [30].

Aggregation experiments. An aqueous suspension of latex beads (Sigma polystyrene latex beads SD 26-diameter 21.1 mm) was added to ciliate cultures (1:500 ml). Control experiments consisted of latex beads incubated in sterile culture medium. As the experiment progressed, samples were observed under a Zeiss Stemi s V6 or a Nikon SMZ-2T stereoscopic microscope.

Determination of EPS composition. Three different lectins were tested for their abilities to stain carbohydrates: peanut agglutinin (PNA), from *Arachis hypogaea*, (Sigma L7381), which reacts specifically with D-galactose; concanavalin A (Con A), from *Canavalia ensiformis*, (Sigma C7642), which reacts with D-manose and D-glucose; and wheat-germ agglutinin (WGA), from *Triticum vulgare*, (Sigma L4895), which reveals *N*-acetyl-D-glucosamine residues. Each of the lectins was coupled to fluorescein. Working and stock solutions were prepared following the procedures of Wilks and Sleigh [32]. Axenic cultures of *T. thermophila* were incubated with latex beads (1:500 ml) and 50 mg of each type of lectin/ml for 20 min and then washed three times with phosphate-buffered saline (PBS). The absence of unspecific binding to the latex beads was confirmed by controls with each of the carbohydrates. Lectins were also assayed by incubation with ciliate cultures (200 cells/500 ml) without the addition of latex beads. Ciliate samples were treated with 5-[(4,6-dichlorotriazin 2-yl]amino) fluorescein hydrochloride (DTAF) (Sigma D0531) for the detection of proteins, and with propidium iodide (Sigma 81845) to detect DNA of non-viable cells. The cells were double stained by incubating them for 40 min in the presence of both fluorochromes diluted in PBS until a respective final concentration of 2 and 10 mg/ml. Excess stain was washed off with PBS.

Microscopy. Preparations were observed under a Zeiss Axioplan microscope with a refrigerated digital camera (CCD spot Camera). Images were analyzed with the software MetaMorph Imaging System (Universal Imaging System).

Quantitative determination of exopolymers. The quantity of exopolymers produced by the ciliates in axenic conditions was measured in 140 ml of PPG medium plus antibiotic inoculated with 10 ml of a culture in exponential growth phase. Aliquots of these cultures were taken daily from 72 h to 168 h for quantification. Samples were filtered through 0.45-µm pore size filters (Millipore) to remove ciliate cells, and the filtered solution was stored at –80ºC. The exopolymers were characterized quantitatively using different colorimetric methods: polysaccharides were assessed by the phenol/sulfuric-acid method [8]; proteins, by the Bio-Rad protein assay (500-0006) [3]; and uronic acids by the carbazol method [2]. Standard concentrations were expressed as mg/ml. Each of the experiments was carried out in duplicate.

Results

Exocytosis experiments. The exposure of axenic cultures of *T. thermophila* to Alcian blue induced active exocytosis by the cells. Immediately after exposure, the cells secreted a conspicuous ovoid capsule that surrounded most of the ciliates. Within several minutes, cells emerged from the capsule (Fig. 1A), and the capsular residues enhanced the formation of aggregates in the cultures (Fig. 1B).

Aggregation experiments. Aggregation of latex beads was observed as early as 3 h after the addition of particles to axenic cultures of the ciliate. These aggregates settled when mechanical shaking was stopped. After several days, there were many small, loosely compacted aggregates, with open spaces between particles (Fig. 2A). Control experiments of latex beads in PPG medium showed no aggregation (Fig. 2B).

Characterization and quantification of exopolymers in axenic cultures of *T. thermophila*. Analyses of the exopolymeric matrix of axenic cultures are shown in Fig. 3 (A–H). A heterogeneous matrix that strongly stained with the lectin Con A developed over the surface of the particles, which contributed to their binding to each other (Fig. 3A). Axenic cultures with PNA-treated latex beads had a peripheral matrix that stained very faintly and which included more intensely fluorescent rounded structures (Fig. 3B). WGA-treated beads also formed round structures, which were intensely fluorescent and embedded in a faint matrix (Fig. 3C). No specific staining was observed in controls of lectins and latex beads (Fig. 3D). Cellular binding sites were determined by incubating the cells with each of the three lectins. In every case, after 20-min of exposure, *T. thermophila* showed variable labeling of cellular food vacuoles (Fig. 3E,F). Images obtained with DTAF, either from control

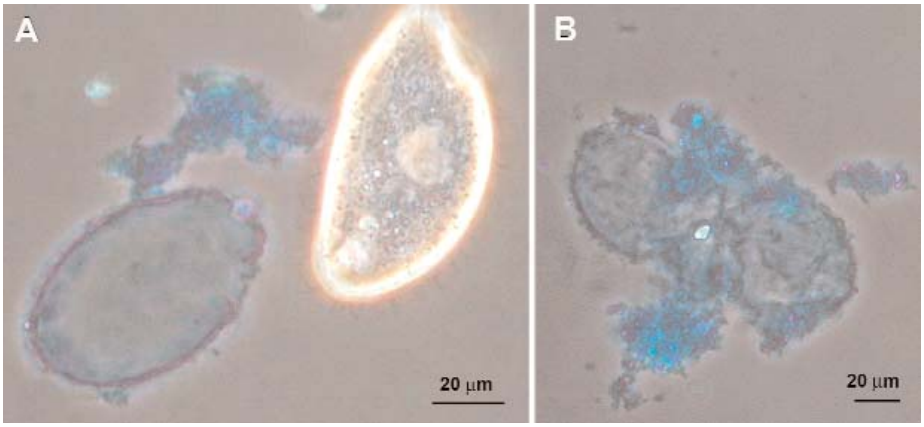

Fig. 1. (**A**) Capsule of *Tetrahymena thermophila* after exposure of the cells to Alcian blue. An emerged cell can be seen adjacent to this structure. (**B**) Aggregate formed by two capsules linked to materials from the culture.

experiments (latex beads in culture medium) or in preparations containing ciliates, did not show any fluorescence (Fig. 3G). However, the nuclei of the ciliates were clearly visible when stained with propidium iodide (Fig. 3H).

Spectrophotometric analysis showed a slight increase in the quantity of exopolymers contained in ciliate cultures (Fig. 4). Polysaccharides were the most abundant polymeric substances detected (Fig. 4). The amounts of proteins and uronic acids hardly changed throughout incubation of the ciliate cultures and did not differ greatly from those of control values (culture medium).

Discussion

The quality of the effluent released from biological reactors of WWTPs depends on the effective development of bioaggregates. These are made up of diverse polymeric substances originating from the biological activity of resident microorganisms. Although initially, production of the intracellular storage product poly-β-hydroxybutyric acid was thought to be responsible for bacterial aggregation, it is now known that EPS, produced by activated-sludge microorganisms, are mainly responsible for floc formation [16].

While bacteria populations are of great importance in wastewater treatments, ciliates constitute the second most relevant community in these systems [17]. *Tetrahymena thermophila*, a free-swimming ciliate found in the mixed liquor, was used to analyze the contribution of ciliates to floc formation independent of bacterial activity. The extrusion of polymeric material by cellular organelles (extrusomes) in response to diverse external stimuli has been illustrated for different species of ciliates. Following discharge of the extrusomes, the cell remains intact and functional and the organelles are replaced within the same cell shortly thereafter (for a review, see [23]). Extrusomes of *T. thermophila*, which have been described as mucocysts, are very numerous and are arrayed longitudinally within the cell [11,27]. Exocytosis can be stimulated by exposing the cells to dibucaine [29] or Alcian blue [20]. In the present study, Alcian blue chemically stimulated the extrusion of capsules around the *T. thermophila* cells, thereby demonstrating that this mucous material contributes to cellular aggregation and facilitates the adhesion of particles in the culture medium. Therefore, the

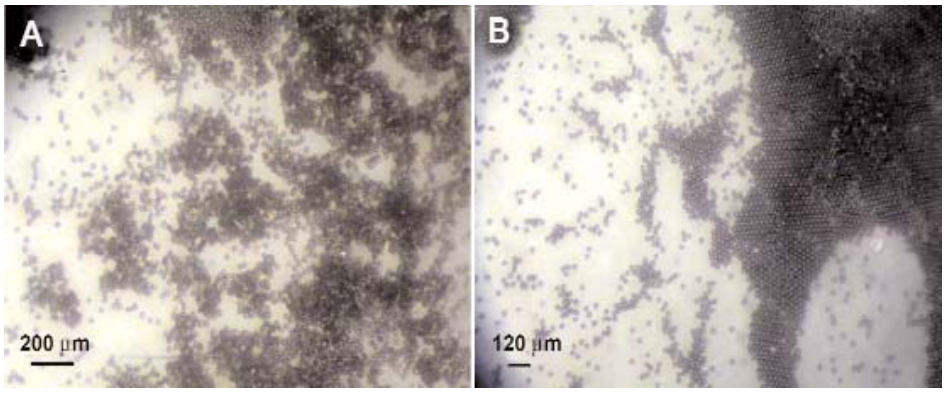

Fig. 2. Aggregation of latex beads in axenic cultures of *Tetrahymena thermophila*. (**A**) Several days after particle addition, numerous small flocs are visible. (**B**) In the control experiment, latex beads accumulated at the bottom of the well with no sign of aggregation.

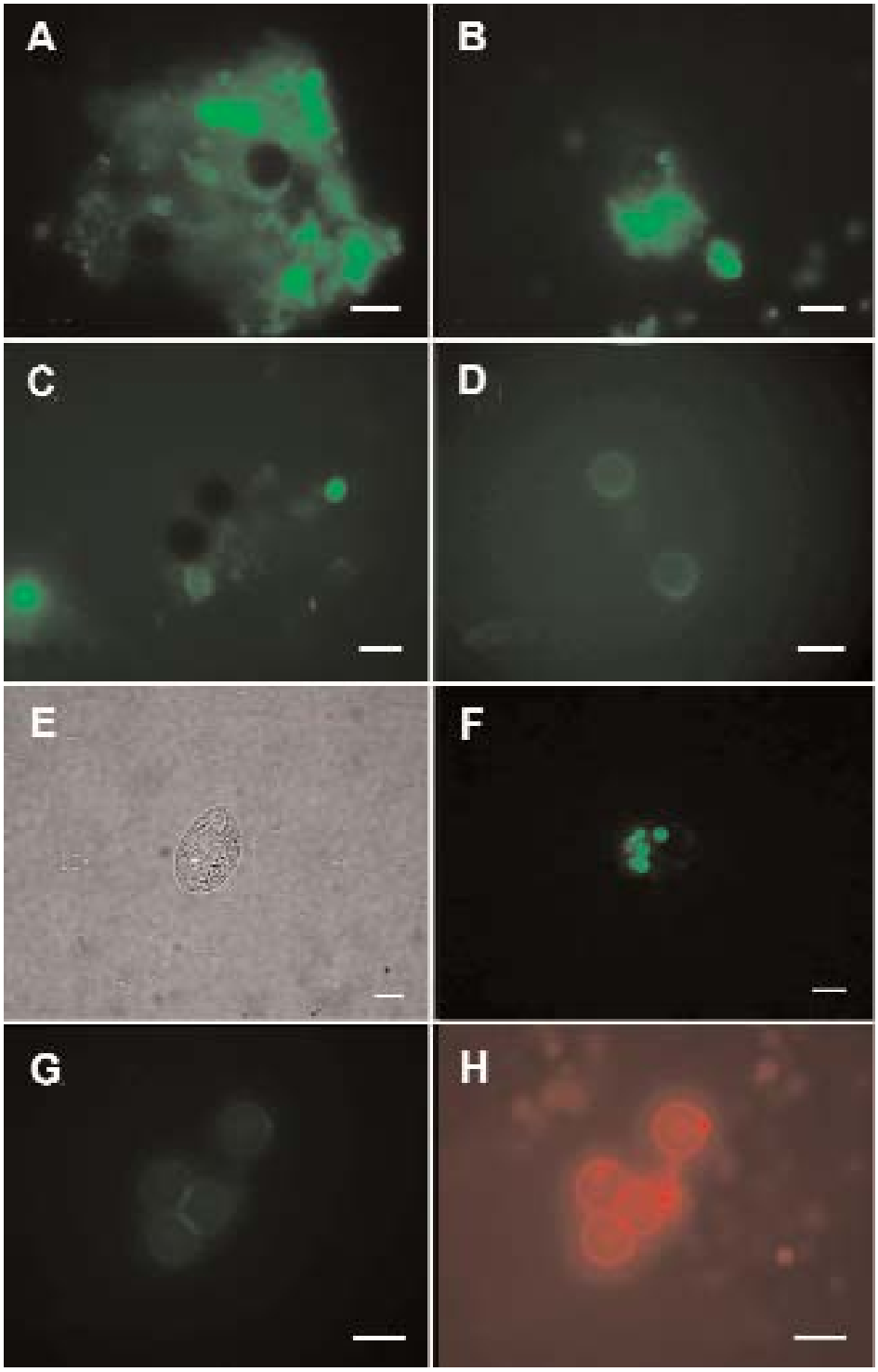

Fig. 3. Aggregates in cultures of *T. thermophila* containing latex beads treated with different fluorochromes. (**A**) Con A stained material. (**B**) Fluorescent staining obtained with PNA. (**C**) Labeling of the material surrounding the beads by WGA. (**D**) Control experiment with a fluorescent lectin. (**E**) Phase-contrast image of a cell after exposure to fluorescent lectins. (**F**) PNA staining of food vacuoles. (**G**) Aggregates stained with DTAF. Fluorescence was not observed around the latex beads added to the ciliate cultures. (**H**) Aggregated latex beads stained with propidium iodide. Fluorescent round structures are attached to the surface of latex beads. Scale bars = 20 μm.

exocytosis of this material is a mechanism by which ciliates—including *T. thermophila*—may contribute to floc formation. Staining of the polymeric material with Alcian blue suggested that it consists of carbohydrates. The addition of latex beads to the cultures revealed the active involvement of *T. thermophila* in the flocculation process, while exposure of the aggregates to diverse fluorochromes allowed the composition of the EPS to be determined. Incubation of the cells with fluorescently coupled lectins showed that the extracellular matrix in axenic cultures of *T. thermophila* comprised glu-

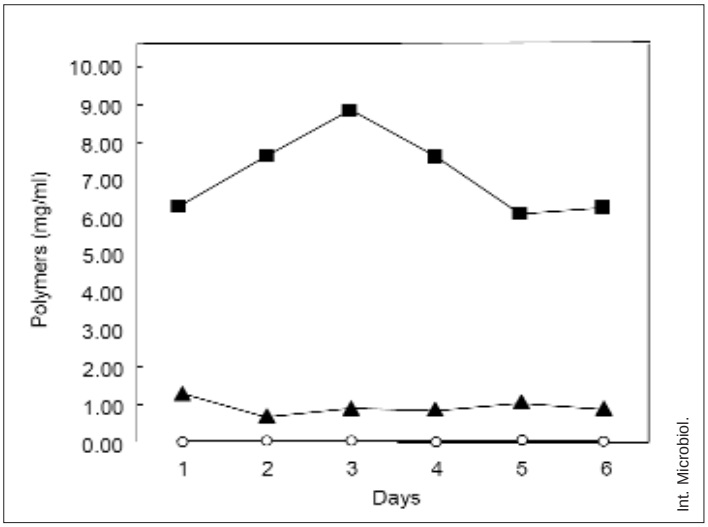

Fig. 4. Proteins (circles), polysaccharides (squares), and uronic acids (triangles) present in the polymeric material were measured over the course of the experiment. Values are expressed in mg/ml.

cose, mannose, *N*-acetyl glucosamine, and galactose (some of the carbohydrates were also detected with the phenol/sulfuric-acid method). The hydrophobic properties of polysaccharides and their negative electrical charges may account for their capacity to promote aggregation by establishing electrical bridges and van der Waals forces [12,14]. Diatoms are other protists able to produce large quantities of polysaccharides, especially glucose and xylose, although the exact constituents differ among species [21,26].

Protein fractions were not detected either colorimetrically or after staining of the matrix with DTAF. However, propidium iodide staining showed that it did contain nuclear residues of ciliates. Although the role of nucleic acids in flocculation is not well-understood, their presence as an important fraction in ciliate cultures suggests that they support floc adhesion by increasing the overall negative charge of the EPS.

In conclusion, the aggregation of particles is observed in axenic cultures of *T. thermophila* exposed to chemical or mechanical stimuli. Ciliate EPS is composed mainly of carbohydrates and nucleic acids, which are involved in matrix formation and the adhesion of aggregates. The contribution of ciliates to bioaggregation may increase the effectiveness of adhesion within aggregates in water purification treatment.

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