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Comamonas testosteroni Colony Phenotype Influences Exopolysaccharide Production and Coaggregation with Yeast Cells

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A *Comamonas testosteroni* strain was isolated from activated sludge on the basis of its ability to coaggregate with yeast cells. On agar plates the following two types of colonies were formed: colonies with a mucoid appearance and colonies with a nonmucoid appearance. On plates this strain alternated between the two forms, making sectored colonies. In liquid medium with constant agitation no such change was observed. In the absence of agitation and in contact with a glass surface a culture with predominantly nonmucoid-colony-forming cells very rapidly shifted to a culture dominated by mucoid-colony-forming cells. In liquid medium the reverse was observed under stress conditions imposed by hydrogen peroxide, sodium dodecyl sulfate, or starvation. Nonmucoid cells formed very rapidly settling flocs with yeast cells, while coaggregation of mucoid cells with yeast cells did not occur. These findings may be relevant to the behavior of activated sludge microbial communities.

Microbial aggregation is an important phenomenon in wastewater treatment systems. In biofilm reactors, as well as in activated sludge systems, a large variety of microorganisms conglomerate in communities with complex interactions. Several mechanisms of aggregation have been proposed to explain how microorganisms are kept together in an activated sludge floc or in a biofilm layer.

The Derjaugin-Landau-Verwey-Overbeek theory (24) states that microorganisms can be considered colloidal particles and that the attractive van der Waals forces are larger than the repulsive electrostatic forces, even at the low ionic concentrations commonly found in municipal wastewater (24). Zita and Hermansson also suggested that the close contact between microorganisms necessary to overcome repulsive electrostatic forces could be facilitated by intraspecies or interspecies lectin sugar binding. This type of surface interaction has been described for autoaggregating yeast cells (15) and also for coaggregating dental plaque-forming oral bacteria (11).

Exopolysaccharide production is generally considered an important phenomenon that is the basis of the formation of stable aggregates and activated flocs (9). Yet too much exopolysaccharide production can influence negatively the ability of activated sludge flocs to settle (23). Indeed, Urbain et al. (23) found that 80% of the variability in the sludge volume index (the volume [expressed in milliliters] occupied by 1 g [dry weight] of sludge after 30 min of settling) in activated sludge plants could be explained by the variation in exopolysaccharide concentration in the flocs and that high sludge volume index values typically are associated with high exopolysaccharide concentrations in the sludge.

Unstable phenotypic expression of exopolysaccharides has been observed in a variety of microorganisms (3, 6, 10). In one *Pseudomonas atlantica* strain, exopolysaccharide production is controlled by insertion and deletion of an insertion element (3), while the conversion of *Pseudomonas aeruginosa* strains to mucoidy is a major pathogenic determinant in the development of cystic fibrosis (for a review see reference 6).

In this article the isolation of a *Comamonas testosteroni* strain that coaggregates with yeast cells is described. Exopolysaccharide production in this *C. testosteroni* strain was found to be unstable, yielding either mucoid or nonmucoid colonies. In liquid medium, environmental factors determined the predominance of either mucoid-colony-forming (MCF) cells or nonmucoid-colony-forming (NMCF) cells.

MATERIALS AND METHODS

Isolation of *C. testosteroni* A20. Strain A20 was isolated from an enrichment culture by using activated sludge supplemented with strain AE815 (22) from a plant treating hospital wastewater (Gent-M plant) as the inoculum. The enrichment medium, which was designed to recover strain AE185, consisted of Schatz mineral medium (19) supplemented with 0.2% azeleic acid (HOOC-C_7-COOH) as the carbon source, kanamycin (300 ppm), rifampin (200 ppm), and cycloheximide (200 ppm). After two cycles of subculturing, 1 ml of the culture was added to threefold-washed yeast cells (strain W303α transformed with multicopy shuttle vector pMA3a) (20) that had been resuspended in 6 mM sodium acetate (pH 4.5)–10 mM $CaCl_2$ to a final optical density at 600 nm (OD_{600}) of 4 (as determined with a double-beam UV/VIS spectrophotometer [Kontron, Zürich, Switzerland]); the final total volume was 4 ml. The microorganisms were allowed to settle for 1 h. The top 3 ml was removed, and the pellet was resuspended in the same buffer. The cells were allowed to settle again. The microorganisms present in the pellet were regrown in the enrichment medium. The bacterial cells in this culture were precipitated again with yeast cells as described above. The bacteria in the pellet were plated onto the same medium to obtain single colonies. It was determined by visual inspection that the isolates were able to coaggregate with yeast cells by looking for aggregate formation in cocultures in YEPD (2% Bacto Peptone [Difco Laboratories], 1% yeast extract, 2% glucose). A 100-μl portion of an overnight bacterial culture grown in Luria broth (LB) (10 g of Bacto Tryptone [Difco] per liter, 5 g of yeast extract per liter, 10 g of NaCl per liter) was inoculated into 5 ml of YEPD that had been seeded with 100 μl of an overnight yeast culture. In addition to AE815 derivatives (some of which had altered surface characteristics [unpublished data]), a variety of other strains were isolated in this way. One strain, strain A20, which coaggregated with yeast cells in a coculture, was characterized by performing API (Biomérieux, Marcy l'Etoile, France), Biolog, and fatty acid methyl ester analyses. The tests were performed according to the instructions of the suppliers of the Biolog and MIDI databases

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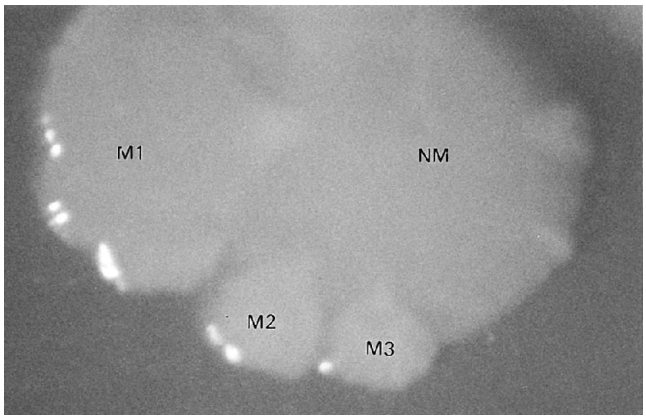

FIG. 1. *C. testosteroni* A20 forming sectored colonies on agar plates. The three large protruding sectors (M1, M2, and M3) were mucoid. The main colony was nonmucoid (NM). The light spots are the result of the reflected light used to make the photograph.

(Biolog, Inc., Hayward, Calif., and Microbial Identification System, Inc., Newark, Del., respectively).

Coaggregation assay with resting cells. *Saccharomyces cerevisiae* W303α (pMA3a) was grown in minimal medium (6.7 g of yeast nitrogen base per liter, 20 g of glucose per liter, 40 mg of adenine per liter, 40 mg of uracil per liter, 40 mg of tryptophan per liter, 40 mg of histidine per liter) overnight and harvested in the early stationary phase. The yeast cells were washed once with MilliQ water and resuspended in 50 mM PIPES [piperazine-1,4-bis(2-ethanesulfonic acid)] (Sigma Chemical Co.) buffer (pH 6.8) containing 5 mM $CaCl_2$ to a final OD_{600} of 2. Bacterial cells that had been grown overnight were washed once with MilliQ water and then added to the yeast suspension to a final OD_{600} of 0.2 while the preparation was vortexed vigorously for 15 s. The total volume of the assay mixture was 5 ml. Formation and precipitation of aggregates were monitored by taking 200-μl samples every 5 min (at the 2.0-ml level) for 30 min. A 800-μl portion of MilliQ water was added to each sample, and the OD_{600} was measured. Coaggregating ability is expressed below as the percentage of residual OD_{600} as a function of time.

Strain A20 was grown either in peptone broth (10 g of Bacto Peptone [Difco] per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter) or in LB. Subcultures were routinely prepared by inoculating 5 ml of medium with 100 μl of an overnight culture and were incubated on a test tube shaker at 28°C at 170 rpm (planktonic growth). In order to obtain sessile growth of strain A20, 5 ml of medium was inoculated with 100 μl of an overnight culture and then put in a lying Roux bottle without agitation.

In the experiments involving chemical stress factors, H_2O_2 (Union Chemie Belge, Ghent, Belgium) or sodium dodecyl sulfate (SDS) (BDH Laboratory Supplies, Poole, England) was added to the medium together with the inoculum. The strain A20 cells were starved in a physiological solution (8.5 g of NaCl per liter in MilliQ water). To do this, 100 μl of a culture containing mainly MCF cells was harvested by centrifugation, washed twice with MilliQ water, resuspended in 10 ml of the physiological solution, and left to stand at room temperature. The A20 cells were treated with proteases (trypsin and proteinase K) as follows. An overnight culture was harvested by centrifugation, washed once with MilliQ water, and resuspended to a final OD_{600} of 1 in 50 mM PIPES buffer containing 5 mM $CaCl_2$. One milliliter of the suspension was incubated with 12 mg of protease for 2 h at 37°C. Treated cells were collected by centrifugation, washed once with MilliQ water, and used in the coaggregation assay.

Exopolysaccharide isolation and characterization. Exopolysaccharide was isolated from the filter-sterilized supernatant (175 ml) of an overnight LB culture that had been inoculated with a mucoid colony. The exopolysaccharide was precipitated overnight at −20°C after 3 volumes of ethanol were added, and the exopolysaccharide was collected by centrifugation. The pellet was redissolved in 30 ml of H_2O, and then the exopolysaccharide was precipitated by adding 3 volumes of ethanol and allowing the preparation to stand overnight at −20°C. This time, precipitation was obtained only after the pH was adjusted to 11 with NaOH. After collection by centrifugation, the pellet was redissolved in 1 ml of MilliQ water and precipitated a third time with 3 volumes of ethanol at a neutral pH. The pellet that was obtained after centrifugation was freeze-dried. The total carbohydrate content of this material was determined as described by Dubois et al. (7), while the uronic acid and hexosamine contents were determined as described by Filizetti-Cozzi and Carpita (8) and Ludoweig and Benmaman (13), respectively. The protein content was determined with Coomassie blue by using the Pierce protein assay reagent (Pierce Chemical Co., Chester, England). Neutral sugars, after hydrolysis with trifluoroacetic acid (final concentration, 2 M; 120°C for 60 min in a capped tube), were derivatized to alditol acetates and analyzed by gas chromatography by the methods of Albersheim et al. (1) and Blakency et al. (4). Samples were analyzed with a Hewlett-Packard model 5890 series II gas chromatograph equipped with a Thames type Rtx-225 capillary column (0.32 mm by 30 m). The carrier gas was helium at a flow rate of 3.0 ml/min. The temperature program was as follows: 180°C for 1 min, followed by an increase at a rate of 2°C/min to 205°C, and then 205°C for 30 min.

RESULTS

Isolation and characterization of *C. testosteroni* A20. On the basis of the ability to coaggregate with yeast cells, a variety of bacterial strains were isolated from an azeleic acid-based enrichment culture seeded with activated sludge. One strain, strain A20, which formed visible aggregates with yeast cells after coculturing for 1 to 2 h in YEPD, was identified as a *C. testosteroni* strain by using API, Biolog, and fatty acid methyl ester tests.

Phenotypic instability. On peptone broth or LB plates, strain A20 formed two types of colonies, translucent and opaque. Further purification by restreaking of both types of colonies always yielded a mixture of the two types. The two types of colonies stained differently in situ with Congo red. The translucent (nonmucoid) colonies hardly adsorbed the dye, while the opaque colonies became dark red, suggesting that the opaque (mucoid) colonies were producing exopolysaccharides (2). The exopolymers that were produced in liquid LB inoculated with a mucoid colony were isolated from the supernatant by ethanol precipitation. These polymers contained 21.3% carbohydrates, 1.1% uronic acids, and no soluble proteins. The carbohydrate fraction contained glucose, arabinose, galactose, ribose, and mannose in the following molar ratio: 1.0:0.1:0.2:0.4:9.0. Some colonies became sectored after 3 to 4 days of incubation (Fig. 1). Translucent colonies with opaque sectors, as well as opaque colonies with translucent sectors, were observed. The sectoring pattern corresponded with the differential ability to adsorb Congo red, with the opaque sectors strongly adsorbing the dye.

Nonsectored colonies from a plate were checked for the ability to coaggregate with yeast cells. An overnight LB culture started with cells of a mucoid colony did not yield yeast-aggregating cells (Fig. 2), as established by the coaggregation assay

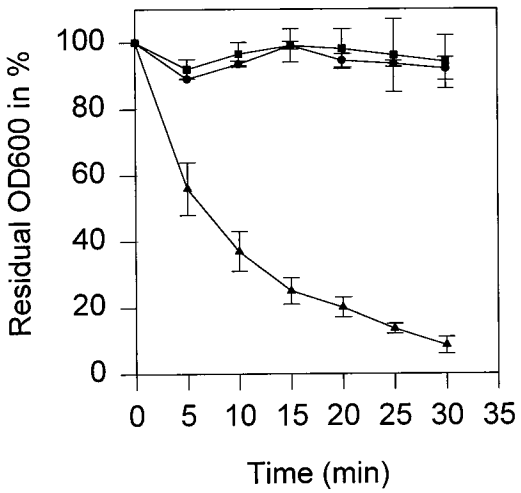

FIG. 2. Coaggregation of A20 cells with yeast cells in the coaggregation assay with resting cells. Symbols: ▲, coaggregation with bacterial cells from an overnight culture started with nonmucoid colonies (mean ± standard error for two individual colonies); ■, coaggregation with bacterial cells from an overnight culture started with mucoid colonies (mean ± standard error for two individual colonies); ●, yeast cells alone.

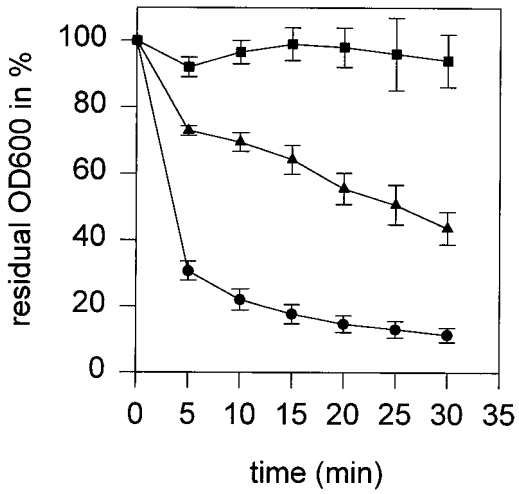

FIG. 3. Coaggregation of yeast cells with A20 cells in the coaggregation assay performed with resting cells: effect of a shift from a culture dominated by MCF cells to a culture dominated by NMCF cells caused by growth in the presence of H_2O_2. Symbols: ■, MCF cells grown in the absence of H_2O_2 (control); ●, NMCF cells obtained by growth in the presence of H_2O_2 (108 ppm) (mean ± standard error for three individual cultures); ▲, NMCF cells obtained by subsequent growth in the absence of H_2O_2 (mean ± standard error for seven assayed subcultures).

with resting cells. In contrast, an overnight culture started with a nonmucoid colony yielded cells able to coaggregate with yeast cells (Fig. 2). Similar data were obtained when this experiment was performed with cells from a mucoid or nonmucoid sector of a colony. These data suggested that in strain A20 phenotypic expression of exopolysaccharide production and coaggregation were unstable and that these two phenomena were linked.

Mucoid-to-nonmucoid shift under stress. The ability of strain A20 to change phenotype during standard laboratory culturing prompted a search for ways to control this process. Hydrogen peroxide stress was tested. A mucoid colony was cultured overnight in LB at 28°C and 170 rpm and then subcultured overnight either in the absence or in the presence of H_2O_2 (108 ppm). The latter overnight cultures were used in the coaggregation assay (Fig. 3). Bacterial cells grown in the presence of H_2O_2 acquired the ability to coaggregate with yeast cells, forming large and fast-settling flocs in the assay mixture. When the preparations were plated to obtain single colonies, the majority (more than 90%) of the cells in each culture yielded nonmucoid colonies. When cells were grown in the absence of H_2O_2, they were not capable of coaggregating with yeast cells and yielded mucoid colonies when they were plated. A shift to NMCF cells could also be obtained by culturing MCF cells in the presence of lower levels of H_2O_2 (e.g., 72 ppm), although less consistently than in the presence of 108 ppm H_2O_2.

The behavior of coaggregating cells upon further subculturing in the absence H_2O_2 was also investigated (Fig. 3). The results indicated that A20 cells maintained the ability to coaggregate, although at a decreased level, even after 10 to 12 subcultures. When cells were plated during these experiments, more than 85% of the cells formed nonmucoid colonies. Two other stress conditions were tested for the ability to induce a shift from a culture dominated by MCF cells to a culture dominated by NMCF cells. Such a shift was observed in cultures containing SDS. Three concentrations of SDS were tested. At 50 or 100 ppm of SDS no effect was observed. In cultures containing 200 ppm of SDS and seeded with 78% ± 2.5% MCF cells, cell growth was strongly inhibited, and there was a 2- to 3-day lag phase. After that lag phase, outgrowth was normal and resulted in a culture containing only NCMF cells (98% ± 1% of the cells). Starvation imposed by resuspending washed MCF cells in a physiological solution could induce a shift. Over a 24-day period the level of MCF cells decreased from 90% ± 2% to 16% ± 3%. When NMCF cells from such a starvation experiment were cultured in LB, they were able to form coaggregates with yeast cells in the coaggregation assay with resting cells and continued to do so even after 10 cycles of subculturing.

Nonmucoid-to-mucoid shift by sessile growth. The development of sectored colonies on agar plates suggested that the change in phenotype was stimulated in the microenvironment of an agar plate and that close cell-to-cell contact or cell contact with a surface could be a trigger. In order to test this hypothesis, individually picked nonmucoid colonies were grown overnight in LB on a test tube shaker and then subcultured daily either with mixing (5-ml LB cultures on a test tube shaker, 170 rpm) or with very poor mixing (static cultures in lying Roux bottles containing 5 ml of LB). Under the latter conditions the culture shifted gradually (over a 3-day period) to a culture dominated by MCF cells (75% of the cells) (Fig. 4). In the intensively mixed control cultures no such shift was observed.

Coaggregation mechanism. Bacterial cells grown overnight in the presence of 108 ppm of H_2O_2 were used to verify the influence of the presence of mannose in the coaggregation assay (Fig. 5). Coaggregation of yeast cells with A20 bacterial cells was found to be very sensitive to mannose; coaggregation was inhibited by 50% in the presence of as little as 10 mM mannose. When protease-treated A20 cells were used in the coaggregation assay, the percentages of residual OD_{600} after 30 min of precipitation were 74 and 89% for trypsin-treated cells and proteinase K-treated cells, respectively, while only 13% was kept in suspension in the control experiment with untreated A20 cells. Also, omitting $CaCl_2$ from the coaggregation test mixture resulted into a strong decrease in coaggregation which left 82% of the initial OD_{600} in suspension after 30 min of settling (compared with 28% in the control). Both

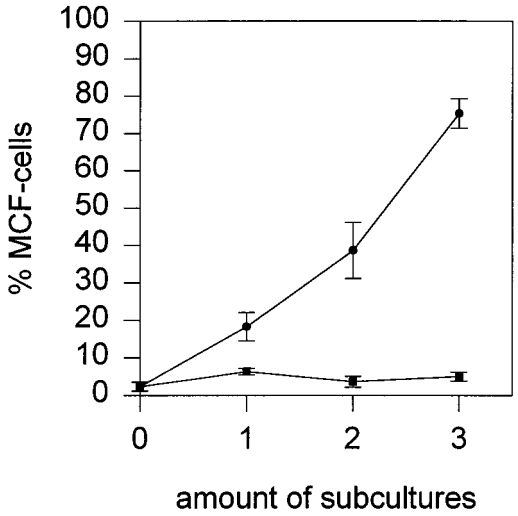

FIG. 4. Shift from a culture dominated by NMCF cells to a culture dominated by MCF cells through sessile growth. Symbols: ■, daily subcultures under mixing conditions (planktonic growth on a test tube shaker); ●, daily subcultures under nonmixing conditions (sessile growth in lying Roux bottles).

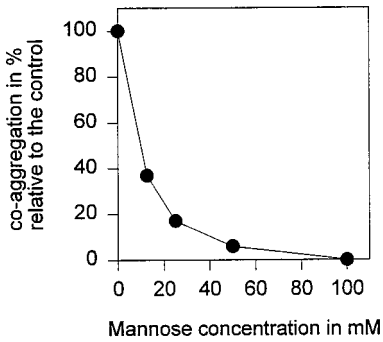

FIG. 5. Effect of mannose on the coaggregation of yeast cells with A20 NMCF cells obtained by subculturing in the presence of 108 ppm of H_2O_2. The level of coaggregation observed after 30 min of settling in the absence of mannose was defined as 100%.

the mannose sensitivity and the protease sensitivity suggested that the coaggregation of strain A20 and yeast cells is based on a lectin-sugar type of surface interaction. This type of aggregation is known to be stimulated by the presence of Ca^{2+} ions (15).

DISCUSSION

C. testosteroni A20 isolated from activated sludge produces two types of colonies on agar plates, opaque mucoid colonies and translucent nonmucoid colonies. Exopolysaccharide produced by a mucoid colony was isolated from a culture supernatant and was mainly composed of mannose and glucose. When cells were cultured and subcultured in continuously agitated liquid media (planktonic growth), they kept their original mucoid or nonmucoid phenotype (Fig. 4). MCF cells grown in the presence of 108 ppm of H_2O_2 yielded a culture containing predominantly NMCF cells (Fig. 3). Upon further subculturing in the absence of H_2O_2, NMCF cells remained the predominant cells. The latter observation strongly suggests that the observed conversion was not due to exopolysaccharide oxidation, which could have taken place initially. Chemical stress imposed by the addition of SDS (200 ppm) could bring about a similar shift. Finally, starving MCF cells also shifted gradually toward NMCF cells. In summary, under environmentally harsh conditions, such as chemical or nutritional stress, the mucoid phenotype was abandoned and NMCF cells became dominant. The reverse shift (i.e., from NMCF cells to MCF cells) was observed only when NMCF cells were allowed to proliferate under nonagitated conditions (sessile growth) (Fig. 4).

Any mechanism to explain the shift from MCF cells to NMCF cells under the stress conditions described above will have to accommodate the fact that in the subsequent absence of the stress factors cells keep the NMCF phenotype as long as they are grown under continuously agitated conditions. It is possible that the combination of stress and agitation is necessary to induce a conversion to nonmucoidy, while agitation alone is sufficient to lock the cells in that state, preventing them from shifting back to the MCF phenotype. It is also possible that classical transcriptional regulation could bring about such phenotypic effects. Recently, in *Pseudomonas aeruginosa*, the AlgU gene, a putative sigma factor, has been shown to be necessary for conversion to mucoidy and for protection against intracellularly generated superoxide radical stress (14). In *C. testosteroni* A20 a similar transcriptional regulator could be at work. Such a regulator could activate the conversion to nonmucoidy in *C. testosteroni* by stress suppressing exopolysaccharide production and could continue to do so as long as cells are grown under continuously agitated conditions. Alternatively, the observed forward and backward shift between NMCF cells and MCF cells could be the result of a genetic switch mechanism. NMCF and MCF cells could be generated in a random way, for which a variety of genetic events (gene conversion, site-specific recombination, oligonucleotide repeat insertion and deletion, homopolymeric tracts, and site-specific methylation) have already been described (16, 18). In this case the environmental stress factors used should select for the better-adapted phenotype. It remains to be established exactly how phenotypic conversion in *C. testosteroni* A20 is brought about.

In the coaggregation test with resting yeast cells, NMCF cells were found to form rapidly settling flocs. This was especially the case when NMCF cells were generated by growth in the presence of H_2O_2. The mannose sensitivity and protease sensitivity of the coaggregation both strongly support the hypothesis that there is a lectin-sugar type of interaction. MCF cells were found not to coaggregate with yeast cells. Either these cells did not produce the lectin-like protein or in these cells the lectin-like protein was present but masked by exopolysaccharides. The latter phenomenon has been described previously for streptococci (21). The current data do not allow us to eliminate either hypothesis.

Aggregation of microorganisms into activated sludge flocs is the result of a variety of phenomena. Most of the microbial surface interactions seem to be brought about by van der Waals attractive forces (24). Intermicrobial binding can be facilitated through protruding specific surface structures and can be consolidated by the production of biopolymers like exopolysaccharides. The stability or cohesion of activated sludge flocs has been found to be controlled by a balance of hydrophobic and hydrophilic interactions (9). The stability of activated sludge flocs is weakened if hydrophilic interactions are the prevailing interactions. Probably as a consequence of this, a high content of hydrophilic exopolysaccharides was found to be correlated with poor sludge settling capacity (23). Strain A20 isolated from activated sludge has both the capacity to surface interact, forming coaggregates with yeast cells, and the capacity to produce exopolysaccharides. In view of the fact that the experimental conditions used in this study are probably considerably different from the in situ conditions, it is hard to predict in which state these types of microorganisms occur in activated sludge and what in situ environmental conditions might regulate exopolysaccharide production in that way. It will be interesting to establish if chemical stress can effectively decrease the amount of exopolysaccharide produced in activated sludge flocs, which contributes to improved settling.

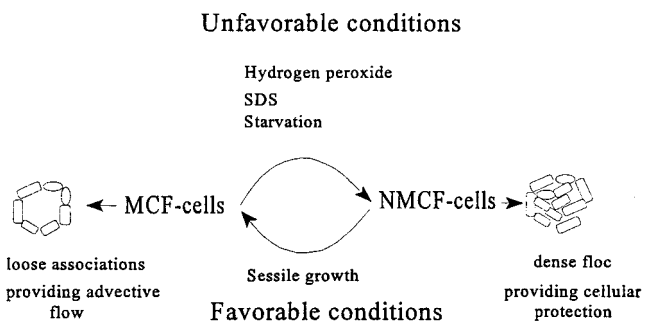

FIG. 6. Scheme summarizing the environmental conditions that allow the forward and backward shift from MCF cell dominance to NMCF cell dominance and the putative repercussions of this shift on microbial aggregates.

With regard to this, it could be that the positive action of oxidative agents (like hypochlorite or hydrogen peroxide) on the settling characteristics of activated sludge flocs (17) can in part be explained by a reduction in exopolysaccharide production.

Assuming that the two phenotypic states (MCF cells and NMCF cells) can occur within an activated sludge floc, it is possible to envisage the following ecophysiological consequences (Fig. 6). When MCF cells are the predominant cells in the floc, it is conceivable that through exopolysaccharide production loose associations are predominant. These loose associations would allow for a channel-rich floc type susceptible to advective flow, as suggested by Logan and Hunt (12) and recently proposed for single-species biofilms (5). Under stress conditions NMCF cells would be the predominant cells in flocs, forming dense conglomerates that provide some protection against cell-penetrating chemicals, such as oxidants.

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