Biotechnology Letters, Vol 20, No 1, January 1998, pp. 1–4

Metabolic blocking of exopolysaccharides synthesis: effects on microbial adhesion and biofilm accumulation

Magali C. Cammarota[1] and Geraldo L. Sant'Anna Jr.[2]*

Instituto de Química[1], Escola de Química[1] and COPPE[2] Universidade Federal do Rio de Janeiro Rio de Janeiro Brazil. P.O. Box 68502, CEP 21945-970, e-mail: lippel@peq.coppe.ufrj.br

A blocking agent of polysaccharide synthesis (5×10^{-4} M 2,4-dinitrophenol) was continuously added to a reaction system, where a heterogeneous microbial population was cultivated at a dilution rate of 0.1 h^{-1}. The results indicate that adhesion and biofilm accumulation were severely reduced when exopolysaccharydes synthesis was blocked.

Introduction

The assumption that presence of capsules or slimes on bacteria would be a prerequisite for adhesion of these organisms on surfaces is attractive and conceivable. However, its acceptability as the determinant mechanism for bacterial adhesion is not universal. This assumption has been often adopted (Fletcher and Floodgate, 1973; Sutherland, 1983; Vandevivere and Kirchman, 1993), but sometimes rejected (Dickson and Daniels, 1991; McEldowney and Fletcher, 1986) on the basis of the findings that many bacteria are wholly lacking in demonstrable slime, and yet readily attach themselves to surfaces. Likewise, bacteria rich in capsules do not necessarily do this. Thus, the question of whether capsules or slimes are a necessary and integral part of the bacterial adhesion is a controversial issue that calls for further investigation.

The role of polysaccharides on bacterial adhesion was early investigated by Marshall and Cruickshank (1973), Mukasa and Slade (1973) and McEldowney and Fletcher (1986), using sodium periodate (a compound that oxides carbohydrates). In the course of these works it was remarked that addition of periodate ion to the culture vessel, in which the adhesion surfaces were submerged, resulted in a rapid desorption of cells from these supports. This result suggested that the extracellular material responsible for anchoring the bacteria to the surfaces would be a periodate sensitive polymer, probably of a polysaccharide nature. However, as observed by Sutherland (1983), small amounts of adhesive polymers might be unaffected (as many gel-forming polysaccharides 1,3 linked) while larger amounts of ineffective (adhesion) slime or capsule might be susceptible to periodate treatment, giving false results.

If the adhesion of cells coincides with or follows the synthesis of exopolysaccharides, then blocking the synthetic pathway, at any point, should prevent adhesion. To support these cause-and-effect observation in our study, the polymer synthesis was blocked by a metabolic blocking agent that uncouples oxidative phosphorylation of carbohydrate and the subsequent effects on bacterial adhesion were evaluated.

Materials and methods

Experimental apparatus

The experimental apparatus, presented in Figure 1, was designed based on the works by Bryers and Characklis (1981) and Pedersen (1982). It contained a substrate feed system, a continuous 2-L stirred tank reactor (CSTR), a test section where biofilm accumulation was monitored and a recycle line, assuring flow through the test section. The culture was mixed (mechanical stirrer) and aerated at a specific air flow rate of 1.0 v.v.m. through a sintered glass air distributor. The volume of reaction system (stirred tank + recycle line + test section) was 1,450 mL. All experiments were run at 27 ± 1 °C. The culture medium was stored at 4 °C and continuously pumped to the stirred tank, using a peristaltic pump. The volumetric feed rate was adjusted to reach a fixed dilution rate in the reaction system. The test section used to investigate biofilm accumulation, illustrated in detail in Figure 1, consisted of eight standard glass microscope slides ($76 \times 26 \times 1$ mm) fitted into a Plexiglas holder, forming a pile. The distance between the slides was 6 mm and the flow rate assured an average liquid velocity of 2.0 $cm.s^{-1}$ through the gaps between the slides. The liquid flow was distributed at the inlet of the test section and to avoid cell deposition by

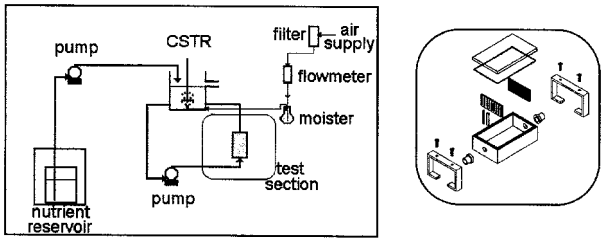

Figure 1 Schematic experimental setup of the reaction system and the test section for biofilm formation (in detail).

gravity and air bubbles on the slides, these were placed in a vertical position. Each slide had a total surface area of 38.48 cm^2 exposed to the flux. The glass slides used in this work were previously washed with hot detergent solution and concentrated nitric acid, rinsed thoroughly with distilled water and oven dried at 100 °C.

Culture medium

The experiments were conducted with a chemically defined medium containing 100 mg glucose.L^{-1}, 36 mg $NH_4Cl.L^{-1}$ and 10 mL mineral salts solution.L^{-1} (Siebel and Characklis, 1991). The culture medium was buffered to pH 7.0 with 10 mM phosphate buffer. To investigate the assumption that adhesion is linked to the synthesis of exopolysaccharides one adhesion experiment was conducted in the reaction system using this chemically defined medium containing 0.092 g 2,4-dinitrophenol.L^{-1} (5×10^{-4} M).

Experimental procedure

The reactor was inoculated with mixed liquor from an activated sludge sewage treatment plant. This inoculum allowed to start the experiments with an heterogeneous culture, typically found in biological treatment units. After inoculation the culture was established by batch growth for 24 hours and then the continuous feed of the tank with culture medium was started at a dilution rate of 0.1 h^{-1}. The reaction system was operated as a continuous cell propagator until suspended biomass, effluent substrate and exopolysaccharide concentrations reached a steady state condition. At that time, defined as zero for that experiment, the slides were fitted into the test section and were exposed to the fluid flow rate. Glass slides were periodically withdrawn to determine biofilm accumulation with the exposure time. During sampling the peristaltic recycle pump was stopped and the test section was removed from the recycle line via two quick-disconnect couplings. The sampled slides were replaced by new ones so that the flow conditions through the pile were maintained. The test section was then replaced in the recycle loop and flow restarted.

Analytical methods

Suspended biomass

Total cell protein was determined in cell suspensions (unfiltered samples) by the method of Lowry *et al* (1951), after cell lysis with an equal volume of 1M NaOH for 5 minutes at 100 °C. Standard protein solutions (bovine serum albumin) were equally treated.

Glucose and extracellular polysaccharide

Samples for glucose and extracellular polysaccaharide analysis were obtained by filtering samples from the reaction system effluent through Millex 0.22 μm pore size filters. Filtrates were frozen until analysis. Glucose was determined by the glucose oxidase method, using the Sigma Chemical Co. test. Polysaccharide was estimated colorimetrically using the phenol-sulfuric acid method of Dubois *et al* (1956) with glucose as standard.

Suspended cell-bound polysaccharide

To analyze suspended cell-bound polysaccharide in the reaction system effluent stream, the following procedure was employed. Effluent samples (50 mL) were filtered in 0.22 μm pore size cellulose ester membrane (Millipore). Bacterial cells retained by the membrane were rinsed with 10 mM phosphate buffer (pH 7.0) and resuspended in 4 mL of 1 mM Na-EDTA dissolved in the same buffer. The mixture was left standing for 2 hours at 4 °C. Filtration through Millex 0.22 μm pore size filters was then performed to remove cells and cellular debris. The filtrate was used to measure suspended cell-bound polysaccharide by the method of Dubois *et al* (1956). This procedure of separation of the cells and polymer adapted from Nishikawa and Kuriyama, as described by Brown and Lexter (1980), stripped the bacteria of most of their capsular material without causing a significant amount of cell lysis as confirmed by low absorbance values measured at 260 and 280 nm.

Biofilm accumulation on glass slides

This parameter was determined by cell protein and attached cell-bound polysaccharide analysis. Biofilm sampling and chemical analysis was performed in the following way: four glass slides were removed at prescribed intervals and gently rinsed with distilled water to remove any loosely attached material. Two slides were used for estimation of the attached biomass: each slide was submerged in 10 mL of 1M NaOH and cells lysis occurred at 100°C for 5 minutes. Cell protein was then determined by the method of Lowry. The other two slides were used to measure the attached cell-bound polysaccharide: the material attached on glass surface was totally scraped off into 50 mL of phosphate buffer, filtered in 0.22 μm pore size membrane and resuspended in 4 mL of 1 mM Na-

EDTA and cell-bound polysaccharide content was measured as above described for suspended cells.

Results and discussion

Results concerning effluent substrate concentration, cell-bound and extracellular polysaccharide contents and biomass (cell protein) concentration in the reaction system operated at a dilution rate of 0.1 h^{-1} with the culture medium without the blocking agent are shown in Figure 2. The residual glucose concentration in the reactor presented very low levels since the initial period of operation, fluctuating in the range of 0.8 to 2.7 $mg.L^{-1}$. An average value of 1.6 $mg.L^{-1}$ was obtained under steady-state conditions, indicating that glucose was almost completely utilized by the microbial population. Extracellular polysaccharide content attained steady-state conditions around the 40th day reaching a value around 3.2 mg glucose equivalent.L^{-1}. Cell-bound polysaccharide concentration practically did not change during period of time of the experiment, showing a mean value of 0.3 mg glucose equivalent.L^{-1}. Cell protein concentration (suspended cells) fluctuated in the range of 2.3 to 8.2 $mg.L^{-1}$ but remained around 2.7 $mg.L^{-1}$ in the last 30 days of operation.

The effect of the addition of 2,4-dinitrophenol to the reaction system on suspended biomass, exopolysaccharides and substrate concentrations can also be seen in Figure 2. After the continuous addition of the blocking agent into culture medium (day 81), effluent substrate concentration was slightly increased. Extracellular and free living cell-bound polysaccharide concentrations did not change significantly, whereas there was a rapid increase on suspended biomass concentration (as cell protein).

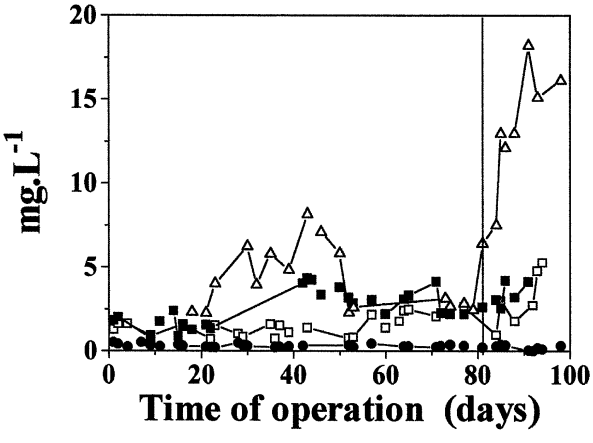

Figure 2 Effect of the addition of 2,4-dinitrophenol on concentration of: (□)residual glucose; (■)extracellular polysaccharide; (●)cell-bound polysaccharide; and (△) cell protein. Lines are provided to illustrate trends. The dot vertical line indicates the start time of continuous addition of 2,4 dinitrophenol to the reaction system.

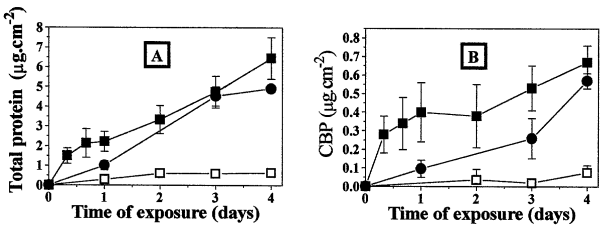

Figure 3 Biofilm accumulation (expressed as biofilm protein content) (A) and cell-bound polysaccharide – CBP (B), on glass surfaces exposed to the medium: (■)without 2,4-dinitrophenol, control; (●)with 2,4-dinitrophenol (slides sampled between days 84 and 92); (□)with 2,4-dinitrophenol, after 16 days of the start of the continuous addition (slides sampled between days 94 and 98). Lines are provided to illustrate trends. Each experimental point is the average of eight replicates (control) or four replicates (tests with 2,4 dinitrophenol).

Extracellular polysaccharide and cell-bound polysaccharide concentrations apparently were not affected by the addition of the blocking agent to the reaction system. However, the specific production of exopolysaccharides (ratio polysaccharides to protein) was gradually reduced attaining very low values around day 91 (0.002 μg cell-bound polysaccharide.μg^{-1} protein). At the same time the specific production of extracellular polysaccharide was 0.228 $\mu g.\mu g^{-1}$. These values were significantly lower than the values observed before the continuous addition of 2,4 dinitrophenol (in the range of 0.050 to 0.147 and 0.474 to 1.385 $\mu g.\mu g^{-1}$, respectively). The results thus obtained for suspended cells indicate that the overall metabolic activity of the mixed culture was mainly drifted for cellular growth and multiplication after starting the continuous addition of the blocking agent (Figure 2).

The effect of 2,4 dinitrophenol on biofilm accumulation (expressed as protein content) is shown in Figure 3A, which also presents the results obtained in the control experiment (without addition of 2,4 dinitrophenol). Biomass accumulation on the slide surface in the control experiment proceeded over a 4 days period of exposure. A biomass level of 6.4 $\mu g.cm^{-2}$ was reached at the end of the experimental period (4^{th} day). Despite the long period of test, steady state conditions were not attained in this experiment. Figure 3A also shows that a drastic reduction on biofilm accumulation was observed when 2,4-dinitrophenol was added to the reaction system. Biomass attained a stable level of 0.6 $\mu g.cm^{-2}$ in the second day of exposure.

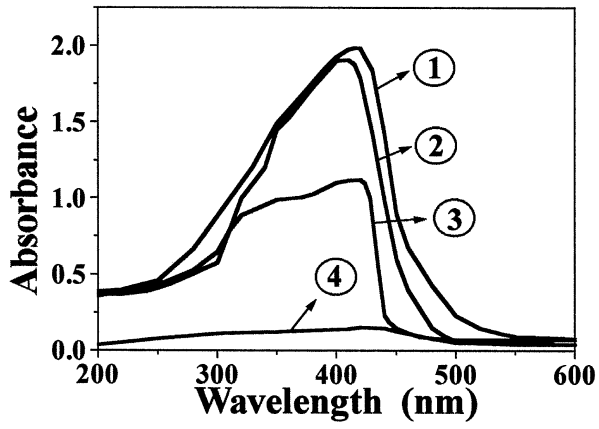

Figure 4 Absorbance spectra of the culture medium with 5×10^{-4} M of 2,4-dinitrophenol (**1**) and reactor effluent after 9 days (**2**), 12 days (**3**) and 16 days (**4**) of continuous addition of 2,4-dinitrophenol.

The results illustrated in Figure 3B show that cell-bound polysaccharide in biofilm is also strongly affected by 2,4-dinitrophenol. Without addition of this compound, cell-bound polysaccharide production increased and reached 0.67 μg glucose equivalent.cm^{-2} by the end of the experiment. Continuous addition of the blocking agent noticeably decreased the cell-bound polysaccharide in biofilm in comparison with control experiment results. Values between 0.020 and 0.076 μg glucose equivalent.cm^{-2} were reached.

The addition of blocking agent significantly affected biofilm accumulation. Comparing levels of both protein and cell-bound polysaccharide in biofilm (Figure 3), it may be observed that, after the continuous addition of the blocking agent, there was an decrease of, at least, 5-fold for protein and 9-fold for polysaccharide contents. These values could confirm the assumption that the adhesion of cells coincides with or follow the synthesis of exopolysaccharides as blocking synthetic pathway prevented the adhesion of cells and the subsequent biofilm formation.

Repetition of the experiments indicated that microorganisms become adapted to 2,4-dinitrophenol, mainly after twelve days of its continuous addition to the reaction system (day 93). Absorbance spectra of the reaction system effluent sampled after 9 (day 90), 12 (day 93) and 16 (day 97) days of continuous addition are presented in Figure 4 and compared with the culture medium spectrum (influent of the reaction system). The absorbance spectra indicate that 2,4-dinitrophenol was consumed by microorganisms after 12 days of continuous addition because its concentration in the effluent was significantly reduced and only compound traces were detected after 16 days. If the addition of the blocking agent caused very low bacterial adhesion, this effect should be reduced when its microbial consumption occurred. This fact was observed in the experiments carried out after 16 days (slides sampled between days 94 and 98) and illustrated in Figures 3A and 3B. As illustrated in these figures, biofilm accumulation (expressed as protein and cell-bound polysaccharide, respectively) increased attaining at the end of the experiment values which were close to those obtained in the control experiment.

In conclusion, the addition of a chemical reagent that uncouples oxidative phosphorylation of carbohydrate prevented both synthesis of polysaccharide and adhesion. This result indicates that there is a direct relationship between the amount of exopolysaccharide produced and adhesion in the multiple-species biofilm investigated in this work.

References

Brown, MJ and Lexter, JN (1980). Appl. Environ. Microbiol. 40:179–185

Bryers, J and Characklis, W (1981). Water Res. 15:483–491

Dickson, JS and Daniels, EK (1991). J. Ind. Microbiol. 8:281–284

Dubois, M, Gilles, KA, Hamilton, JK, Rebers, PA and Smith, F (1956). Anal. Chem. 28:350–356

Fletcher, M and Floodgate, GD (1973). J. Gen. Microbiol. 74:325–334

Lowry OH, Rosebrough, NJ, Farr, AL and Randall, RJ (1951). J. Biol. Chem. 193:265–275

Marshall, KC and Cruickshank, RH (1973). Arch. Mikrobiol. 91:29–40

McEldowney, S and Fletcher, M (1986). J. Gen. Microbiol. 132:513–523

Mukasa, H and Slade, HD (1973). Infection and Immunity 8:555–562

Pedersen, K (1982). Appl. Environ. Microbiol. 43:6–13

Siebel, MA and Characklis, WG (1991). Biotechnol. Bioeng. 37:778–789

Sutherland, IW (1983). CRC Crit. Rev. Microbiol. 10:173–201

Vandevivere, P and Kirchman, DL (1993). Appl. Environ. Microbiol. 59:3280–3286

Received: 9 September 1997
Revisions requested: 26 September 1997
Revisions received: 24 October 1997
Accepted: 25 October 1997