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Relationship Between Physiological Status and Formation of Extracellular Polysaccharide Glycocalyx in *Pseudomonas atlantica*

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Marine pseudomonads, such as *Pseudomonas atlantica*, are readily isolated from sediments. These organisms form extracellular polysaccharide polymers (glycocalyx). The factors affecting the composition and amount of glycocalyx in batch culture of these organisms were examined. The formation of glycocalyx was stimulated by the inclusion of galactose as the carbon source and by increased surface area resulting from addition of sand to the medium. The composition of the glycocalyx changed during the growth cycle, with a marked increase in the proportions and absolute amounts of uronic acids as the rate of synthesis increased. In estuarine sediments, the glycocalyx contained a carbon content at least as great as in the microbes themselves. The greatest accumulation of these polymers occurred late in the stationary phase when the physiological status of the cells, as measured by the adenylate energy charge, showed maximal stress. Maximal formation of glycocalyx possibly could be used as an estimate of the nutritional status of these microbes.

Recent studies have indicated an increasing importance of the extracellular polysaccharide polymers, the glycocalyx, in microbial ecology (7, 9, 14). In the oligotrophic environment, microbial growth on surfaces is particularly important (18, 21; D. C. White, Symp. Soc. Gen. Microbiol., in press) since surfaces concentrate nutrients. These glycocalyx polymers are critical in the attraction and irreversible binding of marine periphytic microorganisms to surfaces (4–6, 9, 13, 22). The formation of glycocalyx has been shown to be particularly important in heat transfer resistance generated by the marine microfouling films on metal surfaces exposed to running seawater (1, 25, 27) and to the stability of soils and sediments (16, 17, 29, 37). These polymers can condition the local environment around the attached microbes by concentrating nutrients and ions or by facilitating the creation of reducing conditions in aerobic environments. They also protect organisms from toxins, biocides, lytic phages, phagocytosis, or *Bdellovibrio* attack (7, 14).

Many electron micrographs from environmental samples show polymers that stain with ruthenium red, which stains polyanionic polymers. The uronic acids are a unique anionic component of polysaccharides found external to the cellular cytoplasmic membranes (11). An assay was developed in which the polymers containing uronic acids could be recovered quantitatively. The assay involves quantitative methylation of the uronic acids, followed by the reduction with sodium borodeuteride of the esters to alcohols while the esters are still in the polymers. This is followed by hydrolysis, derivatization, and subsequent analysis by gas chromatography-mass spectrometry (11). With this assay, it becomes possible to show a direct correlation between the critical erosion velocity of sandy sediments and the galacturonic acid content of the exopolymer glycocalyx (A. R. M. Nowell, D. Thistle, D. J. Uhlinger, and D. C. White, unpublished data).

To begin to understand the dynamics of the formation and metabolism of these important polymers, we isolated sedimentary microbes that produce mucoid colonies and examined them as monocultures in laboratory experiments. The effects of nutrient composition, temperature, oxygen concentration, and pH on the yields of glycocalyx from several bacterial monocultures have shown different optimal conditions for different isolates (15, 23, 28, 30, 31, 35, 36). In batch cultures of many strains of *Pseudomonas* and *Zoogloea* species, maximal accumulation of glycocalyx appears in the stationary phase of growth (10, 28, 32, 35, 36).

In this study, the physiological status of marine sedimentary *Pseudomonas* monocultures was related to the production and composition of glycocalyx. The physiological status of the

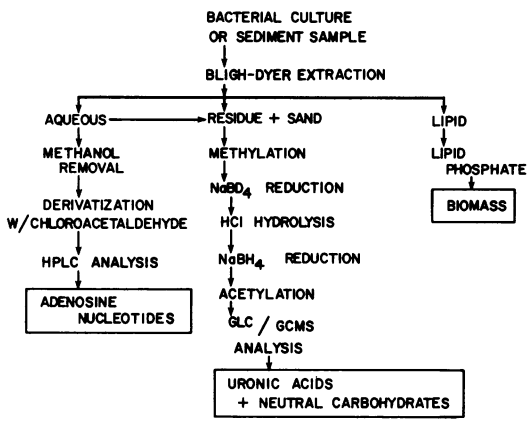

FIG. 1. Diagram of the analytical scheme.

microbes was defined by the adenosine nucleotide energy charge. By measuring all of the adenine-containing components extracted from the cell sap, it is possible to further define one of the homeostatic mechanisms by which the intercellular adenylate energy charge is maintained (8). The activation of this homeostatic mechanism to compensate for falling rates of ATP synthesis gives a more sensitive measure of the physiological status than does the energy charge itself (11). The period when this homeostatic compensatory mechanism was most active correlated with the maximal production of uronic acid-enriched extracellular glycocalyx.

MATERIALS AND METHODS

Materials. Glass-distilled solvents were used as purchased (Burdick and Jackson, Muskegon, Mich.) or freshly distilled just before use in extractions and derivatizations. Lipid standards and derivatizing reagents were purchased from Pierce Chemical Co., Rockford, Ill., Aldrich Chemical Co., Inc., Milwaukee, Wis., and PCR Research Chemicals, Inc., Gainesville, Fla. High-pressure liquid chromatography-grade monobasic ammonium phosphate was purchased from Mallinckrodt Inc., St. Louis, Mo. Sodium borodeuteride (98 atom %D) was obtained from Merck & Co., Inc./Isotopes, St. Louis, Mo. Sand that had been acid washed, ignited, and screened to contain grains between 125 and 175 μm was purchased from Fisher Scientific Co., Pittsburgh, Pa.

Organisms. *P. atlantica* T6c was the gift of W. A. Corpe, Columbia University, New York. Gram-negative motile rods of similar morphology and biochemical characteristics were isolated from sterilized glass slides inserted into estuarine sediments for 3 days and then washed with sterile seawater five times. The adherent colonies were transferred to agar plates made with 1% (wt/vol) galactose and 0.5% proteose peptone (Difco Laboratories, Detroit, Mich.) in 250 g of sea salts (Instant Ocean; Aquarium Systems Inc., East Lake, Ohio) per liter. Cultures were maintained on this medium at room temperature.

Growth of organisms. One-hundred-milliliter portions of sea salts medium containing 1% galactose and 0.1% (wt/vol) proteose peptone were placed in 15 500-ml Erlenmeyer flasks, each of which contained 50 g of the acid-washed, ignited sand. Each flask was inoculated with 1 ml of an exponential culture of *P. atlantica* and incubated at 25°C with shaking at 150 rpm. At 0.2, 1, 2, 4, and 8 days, three flasks were removed, 5-g samples of sediment were recovered with a wide-mouth pipette for adenine nucleotide analysis, and the rest of the sand and medium was utilized for analysis of the lipids and exopolymer glycocalyx.

A diagram of the analytical sequence is given in Fig. 1.

Lipid extraction. For the determination of total adenosine nucleotides, the sediment-medium sample was added to a modified chloroform-methanol-EDTA-phosphate buffer mixture and extracted within 15 min (8). The aqueous phase was recovered for analysis of the adenosine nucleotides. The extracellular adenosine nucleotides were determined by filtering a portion of the sediment suspension through a 0.2-μm filter with gentle suction. The filter was rinsed three times, each time with 5 ml of sterile artificial seawater, and the combined aqueous portions were extracted as described above. Sediments remaining in each flask were extracted by the modified Bligh and Dyer (2) single-phase chloroform-methanol method (34), the phases were separated by adding additional buffer and chloroform, and the chloroform phase was filtered through fluted, folded Whatman 2V filter paper. The lipid-extracted residue plus the precipitated glycocalyx from the medium was lyophilized.

Environmental samples. On 15 December 1981, sedimentary cores of muddy sediment and sandy sediment were sampled with hand-held corers by divers using SCUBA at the Florida State Marine Laboratory (30° 54.8′ N, 84° 30.5′ W). The top 2 cm of the 5-cm-diameter cores were extruded and passed through a 500-μm sieve before extraction in the field (12). The lipid was saved for phospholipid analysis, and the sediment was saved for exopolymer glycocalyx analysis.

Phospholipid analysis. Portions of the lipid samples were dried in a stream of nitrogen and digested in perchloric acid, and the phosphate was determined colorimetrically (34).

Analysis of carbohydrate exopolymer. The uronic acids in the exopolymer polysaccharides were quantitatively esterified and reduced with sodium borodeuteride before hydrolysis and assay by gas chromatog raphy-mass spectrometry (11). Water was removed from the aqueous phase and recovered from the sediment after lipid extraction by vacuum distillation. The dried material was stirred with methanolic HCl (methanol-concentrated hydrochloride-chloroform, 10:1:1, [vol/vol]) for 24 h at 25°C to form the methyl esters. After neutralization with 1 M $NaHCO_3$, the material was dialyzed overnight against distilled water. The dialyzed material was then lyophilized and reduced by sodium borodeuteride. After hydrolysis, the carbohydrates were reduced to alditols, peracetylated, and separated by gas-liquid chromatography. The proportion of deuterium in each carbohydrate that was a uronic acid in the polymer was determined by gas chromatography-mass spectrometry (11).

Adenosine nucleotide analysis. The adenosine-containing components of the microbial cytoplasm were

TABLE 1. Exopolymer glycocalyx and cellular component composition during growth of *P. atlantica*

Sample[a]	Composition (nmol/g [dry wt] of sand)[b]							
	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Mannuronic acid	Galactose	Galacturonic acid
0.2	6.7 (5.2)^{A}	4.2 (3.0)^{B}	39.8 (13.6)^{C}	47.1 (13.8)^{D}			370 (247)^{H}	40.3 (6.2)^{I}
1	8.7 (4.2)^{A}		32.67 (9.8)^{C}	76.9 (69.9)^{D}	54.1 (26)^{E}		426 (105)^{H}	68.1 (33.1)^{I}
2	48.9 (32)^{A}		55.3 (16)^{C}	52.7 (32.7)^{D}	222 (137)^{E}		645 (372)^{H}	266 (206)^{I,J}
4	204 (145)^{A}		37.3 (29)^{C}	70.7 (4.9)^{D}	743 (488)^{E,F}	239 (146)^{G}	1,620 (740)^{H}	874 (416)^{J}
8	503 (139)	11.4 (0.9)^{B}	20.0 (16.3)^{C}	81.2 (21)^{D}	1,900 (488)^{F}	555 (58)^{G}	3,460 (716)	1,890 (390)
Mud	72 (35)	51 (28)	3.0 (2.2)	45 (25)	61 (38)		53 (32)	6.1 (2.3)
Sand	7.0 (4.0)	7.0 (3.0)	2.1 (1.1)	5.1 (2.2)	8.2 (3.2)	0.01 (0.01)	14 (6.3)	1.0 (1.2)

[a] Numbers represent samples harvested after 0.2, 1, 2, 4, and 8 days; mud and sand sites are estuarine samples.
[b] Mean (± standard deviation); $n = 3$. Values with the same superscript capital letter do not differ at the 0.05 level by Tukey's wholly significant difference test.

TABLE 1—*Continued*

Composition (nmol/g [dry wt] of sand)[b]							
Glucose	Glucuronic acid	Total uronic acid	Total neutral CHO	Total CHO	% Uronic acids	Total adenylates	Lipid phosphate
112 (71)^{K}		49.3 (21.6)^{M}	595 (354)^{O}	644 (376)^{P}	8.1 (1.3)^{Q}		
119 (54)^{K}	47.6 (24)^{L}	116 (56)^{M}	712 (265)^{O}	827 (317)^{P}	13.6 (2.7)^{Q}	0.013 (0.01)	3,200 (1,500)^{T}
577 (351)^{K}	214 (132)^{L}	506 (329)^{M,N}	1,630 (838)^{O}	2,140 (1,160)^{P}	22.5 (3.4)^{R}	0.028 (0.01)^{S}	5,300 (500)^{T}
2,420 (1,730)^{K}	751 (506)^{L}	1,840 (1,060)^{N}	5,100 (3,000)^{O}	6,940 (4,120)^{P}	26.7 (0.5)^{R}	0.022 (0.009)^{S}	4,800 (90)^{T}
5,690 (1,320)	1,630 (356)	4,130 (797)	11,700 (2,500)	15,800 (3,250)	26.2 (1.6)^{R}	0.013 (0.008)	10,700 (1,200)
189 (130)	14 (8.2)	19 (9.1)	529 (110)	548 (230)	3.5 (2.0)		22 (7.1)
61 (33)	4.4 (2.3)	6.1 (4.1)	115 (100)	121 (90)	5.0 (2.0)		24 (2.0)

extracted quantitatively, derivatized, and assayed by high-pressure liquid chromatography (8). The methanol in the aqueous portion of the rapid lipid extraction was distilled in vacuo, and the water was removed by lyophilization. The fluorescent 1:N^6-etheno derivatives of the adenine-containing components were formed by reaction with chloroacetaldehyde. The derivatized components were then separated on a column (30 cm by 4 mm) containing Micropak AX-10 (Waters Associates, Boston, Mass.) with a ternary gradient formed by the Varian 5000 high-pressure liquid chromatograph (modified from reference 8). The three solvents were as follows: A, acetonitrile–5 mM $NH_4H_2PO_4$ (5.7:1 [vol/vol]; pH adjusted to 2.85 with H_3PO_4); B, 5 mM $NH_4H_2PO_4$ (also at pH 2.85); and C, 0.75 M $NH_4H_2PO_4$ (pH 4.5). The liquid chromatograph was programmed for an initial 15-min isocratic run with solvent A. In the next 25 min the chromatograph formed gradients of 100 to 0% A and 0 to 100% B, followed by 40 min of 100 to 0% B and 0 to 100% C and a terminal 10-min isocratic period with solvent C. The column was regenerated by 5-min isocratic runs with solvents C, B, and A. (It is important not to let solvents A and C mix as a salt precipitation occurs.) The data from the fluorimetric detector were interfaced with the Hewlett-Packard 3502 laboratory data system for analysis.

Statistical analysis. The analysis of variance and linear regression were done with program 26-1705 and the Radio Shack TRS-80 computer. Significance of regression lines was determined by a two-tailed *t* test (3). Tukey's wholly significant difference test (24) was run on the population means that maintained the error rate per family at alpha = 0.05. A statement error rate of 0.0045 or less was required before an individual parameter was considered significant.

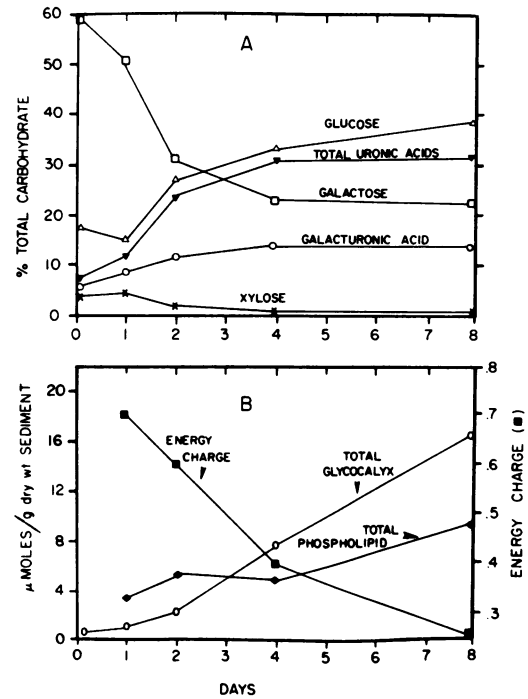

FIG. 2. (A) Changes in the composition of the glycocalyx during the growth cycle of *P. atlantica*. (B) Relationship between the adenylate energy charge (energy charge = [(ATP + 0.5 ADP)/(AMP + ADP + ATP)], the cellular biomass measured as lipid phosphate, and the total glycocalyx during the growth cycle of *P. atlantica*.

RESULTS

Extracellular glycocalyx formation. The yield of the polysaccharide exopolymer from *P. atlantica* grown to stationary phase in the artificial seawater medium with 0.1% (wt/vol) proteose peptone was measured after the addition of 1% (wt/vol) glycerol, xylose, mannose, glucose, or galactose. The highest yield of polymer was produced by the addition of galactose as the major carbon source. Increasing the surface area by adding sand to the medium stimulated the formation of glycocalyx.

Changes in carbohydrate extracellular glycocalyx during growth. Incubation of *P. atlantica* in a seawater medium and sand mixture resulted in a threefold increase in the extractable phospholipid and a twofold increase in the levels of intracellular adenosine nucleotides, which decreased to the initial levels by the end of the experiment (Table 1). During this period, there was a 24-fold increase in the total polysaccharide carbohydrate level. The uronic acid content increased 83-fold, with the uronic acids increasing from 8 to 26% of the total carbohydrates (Fig. 2). In this same growth period, the neutral carbohydrates of the polysaccharides increased 19-fold. These growth conditions induced essentially no change in the total arabinose and xylose contents of the polysaccharides. The carbohydrate composition clearly changed in this period (Fig. 2A). The galactose/galacturonic acid ratio decreased from 9.3 to 1.8, compared with an increase from 2.4 to 3.4 for the glucose/glucuronic acid ratio. This change in composition is reflected in an increase of 0.7 to 3.2% for rhamnose, 6.3 to 12% for galacturonic acid, 17.4 to 36% for glucose, and 1.4 to 11% for glucuronic acid, compared with a decrease of 57 to 22% for galactose of the total carbohydrate.

The formation of polysaccharide components expressed in terms of cellular biomass (measured as extractable lipid phosphate) showed a 16-fold increase in the total uronic acids and an 8-fold increase in the total neutral carbohydrates during the growth period. The galacturonic acid content increased 3 times faster than the galactose content (12-fold increase in galacturonic acid and 3.8-fold increase in galactose). Glucose increased 23-fold compared with a 16-fold increase in glucuronic acid.

Exopolymer from the environment. The top 2 cm of the sediment column of muddy and sandy

TABLE 2. Intracellular and extracellular adenosine nucleotides, adenosine, and adenylate energy charge in *P. atlantica*

Day	Intracellular adenylates (nmol/g [dry wt] of sand)[a]						Extracellular adenylates (nmol/g [dry wt] of sand)[a]			
	Adenosine	AMP	ADP	ATP	Adenylate energy charge	ATP/adenosine $\times 10^4$	Adenosine	AMP	ADP	ATP
1	6.15 (0.71)	<0.0001	0.009 (0.005)	0.007 (0.006)	0.69 (0.18)	11.4 (7.67)	0.35 (0.08)	0.013 (0.01)	0.01 (0.018)	<0.0001
2	12.9 (2.89)	0.004 (0.001)	0.017 (0.002)	0.013 (0.007)	0.62 (0.04)	10.1 (4.98)	0.84 (0.61)	0.008 (0.007)	<0.0001	<0.0001
4	12.2 (4.35)	0.008 (0.005)	0.009 (0.005)	0.005 (0.002)	0.44 (0.095)	3.96 (1.06)	0.42 (0.17)	<0.0001	<0.0001	<0.0001
8	8.27 (0.58)	0.007 (0.004)	0.006 (0.004)	<0.0001	0.23 (0.14)	<0.0001	6.51 (3.54)	0.088 (0.032)	0.022 (0.01)	<0.0001

[a] Mean (± standard deviation); $n = 3$.

estuarine sediments contained polysaccharides with the composition listed in Table 1. Sterile glass slides recovered from the environmental sites after 3 days were extensively washed with sterile seawater. Swabs from the washed glass slides exposed in the environment yielded gram-negative, motile rod-shaped bacteria that formed mucoid colonies on agar slants. These mucoid colony-forming organisms showed biochemical properties similar to those of *P. atlantica*. The compositions of the glycocalyxes from the two sediments did not differ significantly, although the muddy sediment contained some fourfold more polysaccharide. The polysaccharides from the sediments contained lower proportions of galactose, galacturonic acid, and glucuronic acid than did the polymers formed during the growth cycle of *P. atlantica*. Both sediments contained the same biomass of cells, as determined by the phospholipid content. Calculations of the carbon content of the cells, assuming 50 μmol of phospholipid per g (dry weight) (33) and a 50% carbon content per bacterial cell (20), indicate about 220 μg of microbial carbon per g of sediment. The hexose carbons of the glycocalyxes of the mud and sand sediments represented 23.6 and 6.4 μg of carbon per g of sediment, respectively.

Changes in adenosine nucleotides during growth. Shifts in the adenosine nucleotide composition can be used to monitor the physiological status of cells (19). During the period of cell growth, as measured by the increase in phospholipid, the total adenosine nucleotide content doubled and then declined (Table 1). With these changes, there was a steady decrease in the adenylate energy charge that corresponded to the decrease in ATP and to the increases in AMP and ADP as well as in adenosine (Fig. 2; Table 2). As the incubation continued, there was a rapid increase in the excretion of adenosine, AMP, and ADP. The increasing exopolymer total uronic acid content was linearly related to the decreasing energy charge ($r = 0.78$; $P < 0.01$).

DISCUSSION

Composition of glycocalyx. The composition of the glycocalyx produced by *P. atlantica* changed markedly during the growth cycle. There were decreases in the proportion of galactose and large increases in uronic acids with increased ratios of synthesis (Table 1). The fact that the composition of the exopolymer changed during the growth cycle clearly differs from the findings of Williams and Wimpenny for *Pseudomonas* sp. strain NCIB 11264, in which the composition of the exopolymer was independent of the carbon and energy source (35, 36). For these microorganisms, the yield of glycocalyx

increases as the C/N ratio increases (35, 36). Preliminary evidence indicates another difference among the *P. atlantica* used in this study and other pseudomonads in that decreasing the carbon-to-nitrogen ratio increased the yield of glycocalyx.

Sedimentary glycocalyx. *Pseudomonas*-like organisms are by far the most common mucoid colony-forming aerobic bacteria isolated from marine sediments, although marine sulfate-reducing anaerobic bacterial isolates also produce slime (H. L. Fredrickson, unpublished data). Since the composition of the glycocalyx varies in monocultures, it is not possible to define the sources of the glycocalyx in the sediments by the glycocalyx composition. Although muddy sediments contained more glycocalyx per cellular biomass, no statistically significant difference between the proportions of carbohydrate components in the glycocalyx in the mud or sand could be detected. These polymers are an important component of the sediment, with a carbon content at least equivalent to the microbial carbon. This extracellular carbon source possibly provides some 60% of the carbon for deposit-feeding holothurians (D. J. W. Moriarty, Aust. J. Marine Freshwater Res., in press).

Formation of glycocalyx. Studies of the adenylate energy charge show that it is a universal indicator of the physiological status of the cells (19). Cells with a high energy charge are capable of full activity, and cells with a lower energy charge have a depressed metabolic activity. In sediments, it is particularly important to consider only the intracellular adenosine nucleotides in estimating the energy charge, as the excretion of some of these components is one of the homeostatic mechanisms by which the cells maintain the energy charge in the face of falling ATP levels (8). Increasing evidence of metabolic stress (decreased levels of ATP and high levels of AMP and adenosine both inside and outside the cells) correlated with maximal formation of glycocalyx (Fig. 2). The ATP/adenosine ratio has been shown to be a particularly sensitive indicator of microbial stress (8). At the time of maximal glycocalyx formation, there was a markedly increased excretion of AMP and adenosine with no evidence for ATP synthesis. This suggests that the maximal formation of uronic acid-rich extracellular glycocalyx occurs during periods of metabolic stress. If this should prove to be a reasonably universal property of sedimentary microbes, then the formation of glycocalyx containing uronic acids could be used along with the formation of the intracellular endogenous storage material poly-beta-hydroxybutyrate (26) as estimates of the nutritional status of the microbial community. Measures of the nutritional status may be particularly important in estimating metabolic activity in sediments because stimulation artifacts induced by the disturbance necessary for the measurement of the activity may obscure the true activity (White, in press).

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