Bioacetylation of Seaweed Alginate

JIN W. LEE¹ AND DONAL F. DAY^{2*}

*Department of Microbiology¹ and Audubon Sugar Institute,² Louisiana State University,
Baton Rouge, Louisiana 70803*

Received 20 June 1994/Accepted 4 December 1994

Seaweed alginate was acetylated by intact, resting cells of *Pseudomonas syringae* ATCC 19304. Maximum acetylation of this polymer occurred at a pH of 6.0 and a temperature of 25°C. Aeration and gluconic acid were required for an optimal reaction. A reactor which contained carbon-immobilized cells was constructed to continuously acetylate alginate. The maximal yield of acetylation was about 90%, and the half-life of this system was 6.5 days.

Alginates from seaweeds consist of two monomers, β -D-mannuronic acid and its epimer, α -L-guluronic acid, arranged in block structures, which can be homopolymeric (poly- β -D-mannosyluronic acid and poly- α -L-gulosyluronic acid) or heteropolymeric (alternating block structure) (13, 15, 17, 18). The D-mannuronic/L-guluronic acid (M/G) ratios of these alginates are dependent on the algal species as well as the location of the polymer in the plant (13). The M/G ratio forecasts the gelling properties of the polymer. Alginates with low M/G ratios produce strong and brittle gels, whereas alginates with high M/G ratios form elastic gels (22). Alginate-like exopolysaccharides, bacterial alginates, are produced by some bacteria, specifically *Azotobacter vinelandii* (15, 23) and several *Pseudomonas* species (1, 11, 12, 19), including *Pseudomonas syringae* (9). The most conspicuous difference between seaweed alginates and these bacterial polymers is that the mannuronate residues of bacterial alginates are O acetylated (5). The O-acetyl groups are associated exclusively with the D-mannuronic acid residues (24). Detailed analyses of bacterial alginates show that most D-mannuronic acid residues are mono-O acetylated, but a few are 2,3-di-O acetylated.

The biosynthetic pathway for alginate produced by *P. aeruginosa* was described more than two decades ago (3, 19). However, the final steps of biosynthesis, including O acetylation, are still not well understood. The probable source of the acetyl groups is acetyl coenzyme A (acetyl-CoA) (27), but the precursors and mechanism have not been determined. Random acetylation of seaweed alginate has been shown to increase the viscosity and decrease the affinity of these polymers for calcium ions. Acetylated gels also have enhanced swelling ability (25, 26). Specific acetylation of alginates may improve on these and other properties, expanding the commercial potential of this polysaccharide.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *P. syringae* subsp. *phaseolicola* ATCC 19304 was selected for this work because it produces a highly acetylated alginate (9). Cultures were maintained on a modified Dworkin and Foster (DF) agar (8). This medium contained 4 g of potassium phosphate (monobasic) per liter, 6 g of sodium phosphate (dibasic) per liter, 0.2 g of magnesium sulfate 7-hydrate per liter, 0.5 g of ammonium sulfate per liter, 0.4 g of sodium chloride per liter, 9.1 g of potassium nitrate per liter, and 20 g of gluconic acid per liter, and for solid medium, 15 g of agar per liter. The pH of medium prior to sterilization was between 6.7 and 6.9. Starter cultures were prepared by transferring cells from agar slants to 50 ml of modified DF medium with 2% (wt/vol) gluconic acid in 250-ml Erlenmeyer flasks. Cultures were incubated for 30 to 35 h at 30°C and 120

rpm on a model G25-KC rotary shaker (NBS Co., Edison, N.J.) until an A_{650} of between 1.6 and 1.8 was reached.

Production of bacterial alginate and its degree of acetylation. Fructose, gluconic acid, glucose, glycerol, mannitol, and sucrose were tested for the ability to enhance both production and acetylation of the alginate produced by *P. syringae*. The concentration of each carbon source was 2% (wt/vol or vol/vol). After inoculation with 1% (vol/vol) starter culture grown in modified DF medium with 2% (wt/vol) gluconic acid, cultures were incubated for 120 h at 30°C and 180 rpm on an NBS model G25-KC rotary shaker. The amount of alginate produced, its degree of acetylation, and cell concentrations were monitored throughout the growth of culture. Samples were withdrawn over time and then centrifuged at $7,000 \times g$ for 30 min. Pellets were washed twice with distilled water prior to determination of biomass. Supernatants were desalted, either by dialysis for 48 h against distilled water or by being passed through a Sephadex G-25M column (Pharmacia LKB, Uppsala, Sweden). Desalted samples were used for quantitation of alginate and acetyl.

Acetylation of seaweed alginate by resting cells. *P. syringae* ATCC 19304 cells grown in modified DF liquid medium supplemented with 2% (wt/vol) gluconic acid were harvested by centrifugation ($7,000 \times g$ for 30 min) when cell mass reached between 1.3 and 1.4 mg (dry weight)/ml. Cells were washed twice with 0.01 M phosphate buffer (pH 6.8) and then resuspended in the same buffer to a concentration of 13 to 14 mg (dry weight)/ml. Unless otherwise noted, suspensions which contained 2.6 to 2.8 mg (dry weight) of cells per ml were used in all conversions. *P. syringae* cells were incubated at 25°C with 1.5 g of seaweed alginate from *Macrocystis pyrifera* (Sigma Chemical Co., St. Louis, Mo.) per ml and 1.0% (wt/vol) gluconic acid in 0.01 M phosphate buffer (pH 6.8). Samples were withdrawn from this reaction mixture over time and analyzed for amounts of acetyl groups. The optimum aeration, reaction rate, cell age, temperature, pH, gluconic acid concentration, seaweed alginate concentration, and cell concentration for acetylation of seaweed alginate were determined by this assay.

Carbon immobilization of *P. syringae* cells. Stationary-phase *P. syringae* cells were harvested and washed twice with sterile distilled water. Ten grams (wet weight) of these cells was resuspended in 500 ml of sterile phosphate buffer (potassium phosphate monobasic [4.0 g/liter], sodium phosphate dibasic [6.8 g/liter] [pH 6.8]) which contained 25 g of activated carbon (mesh size, 12 by 40 μ m; American Norit Co., Fla.). The mixture was incubated with gentle stirring for 12 h at 4°C. Carbon particles were allowed to settle, the supernatant was decanted, and carbon particles were used in a fluidized bed reactor as the cell catalyst. Immobilization was confirmed by scanning electron microscopy.

Scanning electron microscopy of immobilized cells. Samples were prepared for scanning electron microscopy by washing with 0.1 M cacodylate buffer (pH 7.2) and then fixing for 1 h with 2% glutaraldehyde in 0.1 M cacodylate buffer at room temperature. Fixed samples were washed three times with the same buffer and postfixed for 1 h with 0.8% osmium tetroxide. After a distilled water wash, they were counterstained with 0.5% uranyl acetate. Samples were washed with distilled water and then dehydrated by a graded series of ethanol washes. Dehydrated samples were dried in a DCP-1 critical drying apparatus (Denton Vacuum, Inc., Cherry Hill, N.J.) and coated with platinum by using an S150 sputter coater (Edward Vacuum, Inc., Willington, Mass.). Samples were visualized with a Cambridge S-260 scanning electron microscope.

Acetylation of seaweed alginate by immobilized cells in a fluidized bed reactor. Continuous acetylation of seaweed alginate by carbon-immobilized *P. syringae* cells was tested in a fluidized bed bioreactor fed with 1.5 g of seaweed alginate per liter and 1.0% (wt/vol) gluconic acid in 0.01 M phosphate buffer (pH 6.0). The reactor vessel was a 700-ml airlift bioreactor (Kontes Life Sciences Products, Vineland, N.J.), with a working volume of 500 ml, which contained 25 g of carbon catalyst. The temperature was maintained at 30°C, and the aeration rate was 0.4 standard liter/min (SLPM). Alginate was recovered by alcohol precipitation, and acetylation was quantified by chemical assay.

* Corresponding author.

TABLE 1. Effects of carbon source on growth and production of acetylated bacterial alginate

Carbon source ^a	Cell growth (mg/ml)	Yield ($\mu\text{g}/\text{mg}$ of cells)		Acetylation (%) ^c
		Uronic acid	Acetyl ^b	
Glucose	1.53	199.1	80.0	121
Sucrose	1.46	82.9	2.4	9
Glycerol	1.11	105.3	31.9	91
Gluconic acid	2.03	187.9	109.0	175
Mannitol	0.12	148.5	24.2	49
Fructose	0	0	0	0

^a Cultured in modified DF liquid medium supplemented with 2% carbon source. Cultures were grown for 120 h at 30°C.

^b In bacterial alginate.

^c Ratio in percentage of micromoles of acetyl to micromoles of uronic acid.

Analytical methods. The dry weights of cells were determined by direct measurement of biomass after drying to constant weight at 100°C. Standard curves for alginate and acetyl were prepared from alginic acid sodium salt and glucose pentaacetate (Sigma Chemical Co.) respectively. Seaweed alginate used in these experiments was from *M. pyrifera*; a 2% (wt/vol) solution at 25°C had a viscosity of 14,000 CP. Alginate (uronic acid) concentrations were determined according to the method described by Blumenkrantz and Asboe-Hansen (2). The degree of acetylation was measured according to the method described by McComb and McCready (21). This method is specific for the secondary acetyl groups at 2' and 3' positions of hexouronate residues.

RESULTS

Production of bacterial alginate. *P. syringae* ATCC 19304 cells produced various concentrations of acetylated alginate, depending on the available carbon source (Table 1). Glucose-grown cells produced the most alginate, whereas gluconic acid-grown cells produced the most highly acetylated alginate. *P. syringae* cells grew on glucose and sucrose, but long lag periods (>100 h) were required prior to an increase in cell number. Because of this long lag, gluconic acid was the carbon source of choice. Growth was poor on mannitol. *P. syringae* ATCC 19304 cells did not utilize fructose as the sole carbon source.

P. syringae ATCC 19304 flask cultures produced $380 \pm 20 \mu\text{g}$

FIG. 2. Effects of alginate and gluconic acid on acetylation and polymer production in the presence of seaweed alginate. *P. syringae* ATCC 19304 cells were cultured at 30°C in modified DF medium supplemented with various concentrations of gluconic acid and 800 μg of seaweed alginate per ml. Samples were taken 5 days postinoculation.

of bacterial alginate per ml which contained about $220 \pm 20 \mu\text{g}$ of acetyl per ml after 120-h culture in modified DF liquid medium supplemented with 2% gluconic acid. The apparent amount of acetyl groups per mg of cells remained constant throughout cell cultivation, while the amount of bacterial alginate per mg of cells increased with time (Fig. 1).

When *P. syringae* ATCC 19304 cells were cultivated in the presence of seaweed alginate (800 $\mu\text{g}/\text{ml}$) and gluconic acid, there was no increase in uronic acids, indicating an absence of bacterial alginate production. The acetyl concentration in the polymer increased, depending on the starting concentrations of gluconic acid in media (Fig. 2). It appears that *P. syringae* neither hydrolyzes seaweed alginate nor produces a significant concentration of bacterial alginate when seaweed alginate is present.

FIG. 1. Specific yields of bacterial alginate and acetyl by *P. syringae* ATCC 19304 cells. Cultures were grown in shake flasks at 30°C in modified DF medium supplemented with 2% gluconic acid. In Fig. 1 through 6, error bars show standard deviations ($n = 3$).

FIG. 3. Effects of aeration on the acetylation of seaweed alginate by resting *P. syringae* ATCC 19304 cells at 25°C. Each reaction mixture contained 1.5 g of seaweed alginate per liter, 1.0% gluconic acid, and 2.6 to 2.8 mg (dry weight) of *P. syringae* cells per ml in 0.01 M phosphate buffer (pH 6.8). Acetylation was determined after 10 h.

FIG. 4. Time course for the acetylation of seaweed alginate by resting *P. syringae* ATCC 19304 cells at 25°C with 1.5 g of seaweed alginate per liter and 1.0% gluconic acid in 0.01 M phosphate buffer (pH 6.8). The aeration rate was 0.15 SLPM, and the cell concentration was 2.6 to 2.8 mg (dry weight)/ml.

Acetylation of seaweed alginate by resting cells. For a fixed amount of cells, the degree of acetylation of seaweed alginate increased with aeration. Aeration was required to attain maximum acetylation of alginate by resting cells (Fig. 3). Acetylation reached a plateau at an aeration rate of 0.13 SLPM. All further experiments were conducted at an aeration rate of 0.15 SLPM. The optimum temperature for the reaction was 25°C, and the optimum pH was 6.0. As the pH rose above 6.0, the amount of acetyl on seaweed alginate decreased rapidly; at pH 7.6, there was no acetylation. The time course for acetylation of seaweed alginate by resting cells showed that the rate of acetylation was linear for approximately 12 h after the reaction started (Fig. 4). The specific yield of acetyl on the polymer increased in a nonlinear relationship with gluconic acid concentration, reaching a maximum at 1.5% gluconic acid (Fig. 5).

FIG. 5. Effects of gluconic acid on the acetylation of seaweed alginate by resting *P. syringae* ATCC 19304 cells at 25°C. Each reaction mixture contained 1.5 g of seaweed alginate per ml and various concentrations of gluconic acid in 0.01 M phosphate buffer (pH 6.8) for 10 h. The concentrations of gluconic acid were 0, 0.5, 1.0, 1.5, and 2.0%. The aeration rate was 0.15 SLPM, and the cell concentration was 2.6 to 2.8 mg (dry weight)/ml.

FIG. 6. Effects of seaweed alginate concentration on the acetylation of seaweed alginate by resting *P. syringae* ATCC 19304 cells at 25°C. Each reaction mixture contained 1.0% gluconic acid and various concentrations of seaweed alginate in 0.01 M phosphate buffer (pH 6.8) for 10 h. The aeration rate was 0.15 SLPM, and the cell concentration was 2.6 to 2.8 mg (dry weight)/ml.

The rate of acetylation of alginate increased with alginate concentration, reaching saturation at 2,000 μg/ml (Fig. 6). As expected, younger cells were better than older cells at acetylating seaweed alginate. Mid-log-phase cells produced the highest percentage of acetyl on seaweed alginate after a 10-h reaction (Fig. 7).

Acetylation of seaweed alginate in a fluidized bed, upflow reactor which contained activated carbon-immobilized cells. Carbon provided a surface for the growth of bacteria and absorption of inhibitory compounds (4). A fluidized bed reactor which contained carbon-immobilized cells was operated on a feed of seaweed alginate and gluconic acid. *P. syringae* cells attached and grew well on the surface of activated carbon (Fig. 8). After 5 days of continuous operation, the carbon surface in the reactor contained approximately 7×10^6 cells/mm². This was a 25-fold increase in cell number from the start of operation. Acetylation of seaweed alginate reached its highest efficiency (about 90% of seaweed alginate acetylated) 3 days after startup (Fig. 9). Then the rate of acetylation decreased linearly. The half-life of this reactor system was 6.5 days. Acetylation of

FIG. 7. Effects of cell status on the acetylation of seaweed alginate by resting *P. syringae* ATCC 19304 cells. Cells were harvested at different phases of growth and incubated at 25°C with 1.5 g of seaweed alginate per liter and 1.0% gluconic acid in 0.01 M phosphate buffer (pH 6.8) for 5 and 10 h. The aeration rate was 0.15 SLPM.

FIG. 8. Scanning electron micrographs of carbon-immobilized *P. syringae* ATCC 19304 cells just after immobilization (A) and after 5 days of continuous operation (B).

seaweed alginate continued for more than 15 days. A constant level of acetylation could be maintained by varying the feed rate. Improvements in reactor design can be expected to extend the half-life of this system.

DISCUSSION

Primary metabolites are essential for life and the reproduction of cells, and secondary metabolites are produced after

rapid growth ceases. The production of secondary metabolites is extremely dependent on environmental conditions. Alginate production by *P. syringae* ATCC 9304 cells was low at the initial stage of culture, but then the rate of production increased rapidly after culture shifted into stationary phase. Alginate seems to be a metabolite with a mixed production pattern (6). The acetyl contents of alginates produced by *Pseudomonas aeruginosa* show an inverse linear relationship with the specific

FIG. 9. Continuous acetylation of seaweed alginate by *P. syringae* ATCC 19304 cells in a fluidized bed, upflow reactor. The feed stock contained 1.5 g of seaweed alginate per liter and 1.0% gluconic acid in 0.01 M phosphate buffer (pH 6.0). The feed rate was 0.02 mg h⁻¹. The operation temperature was 25°C, and the aeration rate was 0.4 SLPM.

growth rate (16). Unlike the situation with *P. aeruginosa*, acetylation of the alginate produced by *P. syringae* did not parallel alginate production. It seems that the specific yield of acetyl did not change significantly during cell culture. The acetylation pattern was that of a primary metabolite. Acetyl-CoA is reported to be the source of *O*-acetyl groups on the mannosyl residues of xanthan, the polysaccharide from *Xanthomonas campestris* (14). Acetyl-CoA is also the probable source of acetyl in bacterial alginates (27). The degree of acetylation may reflect acetyl-CoA availability, and acetyl-CoA levels are highest during the growth phase.

The amount of bacterial alginate as well as the degree of acetylation of the bacterial polymer produced by *P. syringae* ATCC 19304 varied with the carbon source. The primary route of glucose catabolism in pseudomonads is via the Entner-Doudoroff pathway. Glucose requires two enzymes to enter the Entner-Doudoroff pathway as 6-phosphogluconate in pseudomonads. Glucose can be converted to gluconate by glucose dehydrogenase in *P. aeruginosa* (28). The long time required for glucose utilization can be explained by the following possibilities. One of them may be a requirement for the induction of two enzymes to convert glucose to 6-phosphogluconate, whereas gluconate requires only a single enzyme. Another possibility is the induction of a glucose dehydrogenase or glucose transport system. Gluconic acid was chosen as the carbon source for acetylation of seaweed alginate because gluconate-grown cells rapidly produced highly acetylated alginates. In pseudomonads, gluconate can be continuously oxidized to acetyl-CoA through the Entner-Doudoroff pathway (28). The high degree of acetylation of alginates produced by gluconate-grown cells supports acetyl-CoA as the putative acetyl source for acetylation of alginate.

The composition of bacterial alginate (M/G ratios and degree of *O* acetylation) changes according to the carbon source (20). Studies of nutritional and physical influences on the chemical composition of exopolysaccharide from mucoid and nonmucoid *P. aeruginosa* cells (6) showed that separate systems for alginate biosynthesis and acetylation of bacterial alginate exist. Recently, an alginate modification gene, *algF*, coding for a 28-kDa polypeptide which controls the addition of acetyl groups to alginate in *P. aeruginosa*, was identified (10).

In *P. syringae*, the separation of alginate acetylation from alginate biosynthesis allowed the use of this strain for acetyla-

tion of similar substrates, i.e., seaweed alginates. The alginate from *M. pyrifera* is known to consist of 60% 4-linked mannanuronic acid and 40% 4-linked guluronic acid residues, whereas the alginate from *P. aeruginosa* consists of 80% 4-linked mannanuronic acid and 20% 4-linked guluronic acid residues (29). When seaweed alginate was added at the beginning of *P. syringae* cultivation, it acted like a feedback inhibitor for bacterial alginate biosynthesis. The similar compositions of seaweed alginate and bacterial alginate probably explain this, although it is possible that localized changes in viscosity or osmotic pressure could shut down alginate biosynthesis as it is known to be under the control of a gene from a class of environmentally responsive bacterial regulatory genes (7).

Resting *P. syringae* cells were treated as an enzyme system to define factors which affect the acetylation of seaweed alginate. The optimum conditions for acetylation as determined for resting cells were applied to a reactor for continuously acetylating seaweed alginate. Development of the microbial process for acetylation of seaweed alginate was possible because the acetylation of alginate was independent of the production of bacterial alginate in this strain. The degree of acetylation was controllable by controlling the length of exposure. This meant that it was possible to produce modified alginates with defined acetylation. These polymers will be the basis of further study of structural and conformational effects on polymer properties. Depending on their characteristics, they may have commercial value as substitutes for seaweed alginates.

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