Biosynthesis of Exopolysaccharide by *Pseudomonas aeruginosa*

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In batch cultures of *Pseudomonas aeruginosa*, the maximum rate of exopolysaccharide synthesis occurred during exponential growth. In nitrogen-limited continuous culture, the specific rate of exopolysaccharide synthesis increased from 0.27 g g of cell^{-1} h^{-1} at a dilution rate (D) of 0.05 h^{-1} to 0.44 g g of cells h^{-1} at $D = 0.1$ h^{-1}. The yield of exopolysaccharide on the basis of glucose used was in the range of 56 to 64%. Exopolysaccharide was also synthesized in carbon-limited cultures at 0.19 g g of cell^{-1} h^{-1} at $D = 0.05$ h^{-1} in a 33% yield. Nonmucoid variants appeared after seven generations in continuous culture and rapidly increased in proportion to the total number of organisms present.

Mucoid strains of normally nonmucoid *Pseudomonas aeruginosa* have been found in association with certain pathological conditions of humans, especially respiratory tract infections accompanying cystic fibrosis (3). Such strains apparently emerge in response to antibiotic treatment (14). They can also be isolated from laboratory cultures by using either antibiotic (9) or phage (15) resistance as a selective agent. Both clinically isolated and laboratory-selected mucoid strains revert to nonmucoid forms at a high rate (10). The exopolysaccharide conferring "mucoidness" in these strains is composed of 1,4-linked D-mannuronic acid and its 5-epimer L-guluronic acid and contains O-acetyl groups (5). It is thus similar to the exopolysaccharide produced by *Azotobacter vinelandii* (7) and to the alginates produced by certain species of brown seaweed (4), differing from the latter by containing O-acetyl groups.

In previous studies on mucoid *P. aeruginosa*, the organism has been cultivated on a complex medium (8, 10, 11). The relationship between exopolysaccharide production and the physiology and growth of the organism has not been studied. We report here studies on polysaccharide production by *P. aeruginosa* in batch and continuous culture with both complex media and a glucose-mineral salts medium.

MATERIALS AND METHODS

Bacterial strain and its maintenance. A mucoid strain of *P. aeruginosa*, isolated originally from the sputum of a cystic fibrosis patient, was obtained from I. W. Sutherland, Dept. of Microbiology, Edinburgh University School of Agriculture, Edinburgh, United Kingdom. To maintain the culture in a mucoid form, it was regularly subcultured in medium A (100 ml) in

a baffled, 500-ml conical flask, grown with gyratory shaking at 30°C for 48 h, and stored at 4°C. Cultures used for fermenter growth studies were derived from single-colony isolates from the stock culture.

Culture media. Medium A, which was used for culture maintenance, contained (grams per liter): sodium gluconate, 20; sodium glutamate, 20; Na$_2$HPO$_4$, 3; and MgSO$_4$·7H$_2$O, 0.3. The pH was adjusted to 7.0 with 1 M NaOH.

Medium B contained (grams per liter): glucose, 20; yeast extract (Difco Laboratories, West Molesey, Surrey, U.K.), 6; (NH$_4$)$_2$SO$_4$, 0.6; Na$_2$HPO$_4$, 2; and MgSO$_4$·7H$_2$O, 0.3. The pH was adjusted to 7.0 with 1 M NaOH.

Medium C contained (grams per liter): glucose, 20; (NH$_4$)$_2$SO$_4$, 0.6; K$_2$HPO$_4$, 2.2; KH$_2$PO$_4$, 0.8; NaCl, 0.2; MgSO$_4$·7H$_2$O, 0.2; ZnSO$_4$·7H$_2$O, 0.2 × 10^{-3}; CuSO$_4$·5H$_2$O, 0.2 × 10^{-3}; MnSO$_4$·H$_2$O, 0.2 × 10^{-3}; CoCl$_2$·6H$_2$O, 0.2 × 10^{-3}; FeSO$_4$·7H$_2$O, 0.6 × 10^{-3}; and CaCl$_2$·2H$_2$O, 0.05.

MacConkey agar was used for plate cultures.

Fermentation conditions. An LH Engineering Ltd. (Stoke Poges, Buckinghamshire, U.K.) type 1/1000 laboratory fermentation module fitted with a 2-liter working volume fermentation vessel, modified for continuous culture of the chemostat type, was used. Fermentation was commenced with a 3% (vol/vol) addition of a shake-flask culture derived from a single mucoid colony isolate that had grown on medium A for 18 h. The fermentation pH was maintained at 7.0 by the automatic addition of 1 M NaOH. The temperature was 30°C. Foaming was controlled by the addition of a silicone antifoaming agent. Air was supplied at 1 liter min^{-1}, and the culture was mixed at an impeller speed within the range 400 to 800 rpm, so as to maintain the dissolved oxygen concentration at ca. 20% of saturation.

For continuous culture, medium addition was commenced when the batch culture reached the stationary phase. The dilution rate was adjusted by alteration of the rate of inflow of medium. A steady state was

considered to exist when the concentration of the propan-2-ol precipitate had remained constant for at least three residence times under unchanged environmental conditions and before nonmucoid variants were detected. The growth-limiting nutrient was demonstrated by increasing the concentration of that nutrient by at least twofold in the inflowing medium and by obtaining an increase in cell concentration.

Bacterial dry weight. Bacterial cells and exopolysaccharide could not be effectively separated in the absence of chelating agent because of polymer-polymer and polymer-cell cross-linking by divalent cations. Tetrasodium ethylenediaminetetraacetate was used. This did not cause lysis of the bacterial cells.

Culture broth (40 ml), which was diluted fivefold if highly viscous, was mixed with 5 M NaCl (0.8 ml) and 0.5 M tetrasodium ethylenediaminetetraacetate (0.8 ml) and centrifuged at 36,000 × *g* for 40 min. The supernatant was separated, and the sediment was suspended in distilled water and centrifuged as before. The sediment was dried at 105°C to constant weight, and the bacterial dry weight of the culture broth was calculated.

Exopolysaccharides. The first supernatant obtained during the bacterial dry weight determination (25 ml) was added to propan-2-ol (75 ml), and the mixture was shaken and let stand for 10 min. The resultant precipitate was filtered off by using a preweighed Whatman GF/A filter disk, which was then dried in vacuo at 45°C for 24 h before it was reweighed and the polysaccharide concentration in the supernatant was calculated.

Similarly, the concentration of propan-2-ol-precipitated material in whole-culture broth was determined during routine monitoring of cultures. The weight obtained approximated to exopolysaccharide weight plus bacterial dry weight.

Glucose determination. Glucose was determined by the glucose oxidase method, using the Boehringer Corporation Ltd., GOD/PERID test combination.

Chemical analysis. When samples of cell-free exopolysaccharide were required for analysis, the overflow of the continuous culture was mixed with propan-2-ol. The resulting precipitate was collected, excess solvent was removed, and the precipitate was dissolved in distilled water. The solution was centrifuged at 36,000 × *g* for 40 min, the clear supernatant was added to propan-2-ol, and the resulting precipitate was collected and freeze-dried. Infrared spectra were recorded with samples in potassium bromide pellets by using a Perkin-Elmer model 457 spectrometer. The mannuronic acid/guluronic acid ratio was calculated from the infrared spectra (6) by using a calibration curve constructed from mixtures of polymannuronic acid blocks and polyguluronic acid blocks (13) in known proportions. The O-acetyl content was determined by the hydroxamic acid method of McComb and McCready (16).

Viscosity. Viscosity measurements were made by using a Wells-Brookfield HBT cone and plate microviscometer at a temperature of 25°C. The apparent viscosities were determined over a range of shear rates from 3.75 to 750 s^{-1}, and the consistency index (apparent viscosity at a shear rate of 1 s^{-1}) was obtained by extrapolation of a log-log plot of apparent viscosity against shear rate.

RESULTS

Polysaccharide production in batch culture. Growth of the mucoid strain of *P. aeruginosa* on a glucose-yeast extract medium (medium B) in batch culture resulted in polysaccharide being produced throughout the growth phase (Fig. 1). Growth and polysaccharide production simultaneously ceased as a result of glucose exhaustion. In the exponential growth phase, the specific rate of polysaccharide synthesis was 0.7 g of cell^{-1} h^{-1} at a specific growth rate of 0.27 h^{-1}. Of the glucose supplied to the culture, 48% was converted into polysaccharide. The polysaccharide produced caused the culture broth to be highly viscous; after 25 h of growth, the consistency index of the broth was 7,000 cP. The polysaccharide produced was not utilized by the organism when glucose was exhausted; neither was there degradation of the polymer when the culture was held at 30°C for 50 h after growth had ceased, the culture viscosity remaining constant during this period. At the end of the growth phase, no nonmucoid variants could be detected when the culture was plated onto MacConkey agar.

Polysaccharide production in continuous culture. To investigate the relationship between growth rate and polysaccharide production, the organism was continuously cultured on medium B under nitrogen-limited conditions, at dilution rates of 0.05 to 0.1 h^{-1} (Table 1). Polysaccharide was produced at each dilution rate tested, with a yield of exopolysaccharide from glucose used in the range of 56 to 64%. Both cell concentration and polysaccharide concentration were largely independent of dilution rate, the specific rate of polysaccharide production increasing with increasing dilution rate. The higher specific rate of polysaccharide production at a specific growth rate of 0.27 h^{-1} during the exponential phase of batch growth is consistent with this finding. In all continuous cultures, nonmucoid variants arose and rapidly increased in proportion to the mucoid strain (Table 2). There was a decrease in polysaccharide concentration of the culture corresponding to the increase in nonmucoid variants. The rate of increase in the proportion of nonmucoid variants is consistent with mutation, with a subsequent selective advantage of nonmucoid organisms, as opposed to a change to a nonmucoid state occurring at a constant rate (12).

When an ammonia-limited continuous culture was grown on a simple chemically defined medium (medium C) in place of the yeast extract-
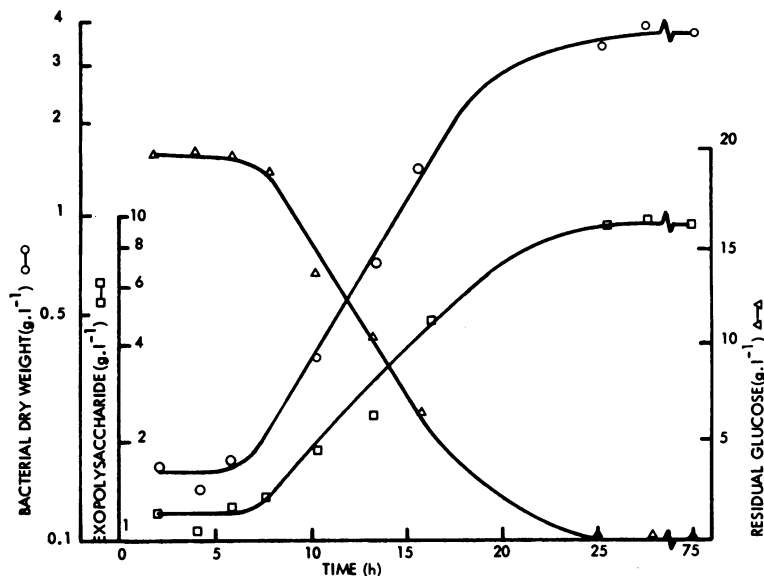
FIG. 1. *Exopolysaccharide production by P. aeruginosa in batch culture. Growth medium B was used.*

TABLE 1. *Exopolysaccharide production by P. aeruginosa in continuous culture*

Dilution rate (h⁻¹)	Bacterial dry wt (g liter⁻¹)	Exopolysaccha- ride concn (g liter⁻¹)	Residual glucose concn (g liter⁻¹)	Exopolysaccha- ride yield from glucose used (%)	Specific rate of ex- opolysaccharide synthesis (g g of cell⁻¹ h⁻¹)
0.05	2.0	10.8	3.3	61	0.27
0.06	2.0	11.3	2.1	63	0.34
0.09	2.3	10.9	2.9	64	0.43
0.10	2.2	9.8	3.6	56	0.44

TABLE 2. *Presence of nonmucoid variants during continuous culture[a]*

Time (h) of continuous cul- ture operation	Nonmucoid colonies as % of total
24	<0.01
48	<0.01
72	5
96	20
112	45

[a] Growth medium B was used with $D = 0.09$ h⁻¹. Culture samples were plated on MacConkey agar. Mucoid and nonmucoid colonies were scored after 48 h of growth at 30°C.

containing medium B, polymer was produced at a similar specific rate and at a similar conversion efficiency from glucose (Table 3). In a carbon-limited steady state, obtained by increasing $(NH_4)_2SO_4$ in medium C, polysaccharide was produced, although at a lower rate than in ammonia-limited cultures.

Nature of the polysaccharide produced. An infrared spectrum of cell-free polysaccharide produced from a continuous culture grown on medium B at $D = 0.05$ h⁻¹ was similar to the

infrared spectrum previously reported for *P. aeruginosa* and *A. vinelandii* polysaccharide (5) and differed from infrared spectra of sodium alginate by containing additional peaks at 1,250 cm⁻¹ and 1,720 cm⁻¹, which correspond to absorption by O-acetyl ester (Fig. 2). The spectrum indicated a mannuronic acid/guluronic acid ratio of 4:1. The O-acetyl content was 5.2% (wt/wt). The polysaccharide produced was therefore considered representative of that produced by a variety of mucoid *P. aeruginosa* isolates as studied by Evans and Linker (5).

DISCUSSION

The majority of studies on exopolysaccharide synthesis by *P. aeruginosa* have involved growth on complex media on agar plates or in shake flasks (10, 11). The strain used on this work, when grown under controlled conditions in continuous culture, produced exopolysaccharide equally well and in high yield both on complex media and on a glucose-mineral salts medium. The observation that nonmucoid variants arise during continuous culture is consistent with previous observations on culture insta-

bility (10, 20). A similar emergence of a nonmucoid strain has been noted with continuous cultures of *Xanthomonas campestris* (1). The rate at which nonmucoid variants of *P. aeruginosa* increase in proportion to the total number of organisms during continuous culture suggests a selective advantage of exopolysaccharide nonproducers compared with metabolically less-efficient producers.

It is apparent that exopolysaccharide synthesis by this mucoid strain of *P. aeruginosa* is a growth-associated process, approximately 5 g

being produced per g of cell mass in nitrogen-limited continuous cultures. The specific rate of exopolysaccharide production thus increased with increased specific growth rate. This differs from exopolysaccharide production by *A. vinelandii, X. campestris,* and another *Pseudomonas* sp. in which the specific rate of exopolysaccharide synthesis has been shown to be largely independent of growth rate (2). The finding that exopolysaccharide is produced under carbon-limited conditions has also been made with *A. vinelandii* (2), which, in common with *P.*

TABLE 3. *Exopolysaccharide production under nitrogen- and carbon-limited growth conditions[a]*

Growth medium	Limiting nutrient	Bacterial dry wt (g liter^{-1})	Exopolysaccharide concn (g liter^{-1})	Residual glucose (g liter^{-1})	Exopolysaccharide yield from glucose used (%)	Specific rate of exopolysaccharide synthesis (g g of cell^{-1} h^{-1})
B	Nitrogen	2	10.8	3.3	61	0.27
C	Ammonia	1.3	8.9	1.6	53	0.34
C'	Carbon	1.5	5.8	0	33	0.19

[a] The dilution rate was 0.05 h^{-1}. To obtain carbon limitation of growth, the ammonium sulfate content of medium C was increased to 2 g liter^{-1}.

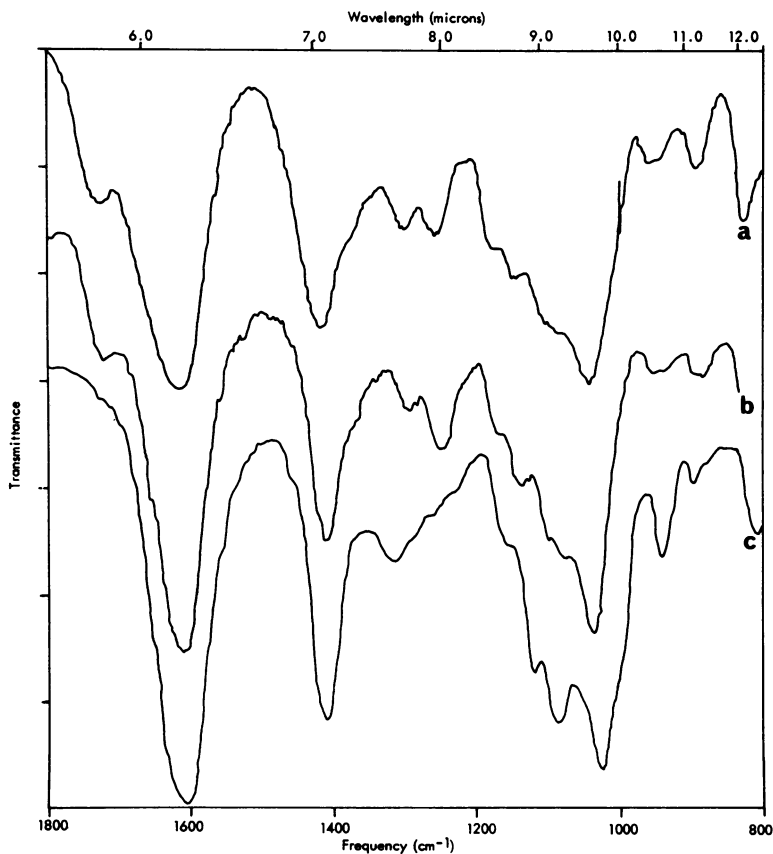FIG. 2. *Infrared spectrum of (a) exopolysaccharide produced by A. vinelandii NCIB 9068, (b) exopolysaccharide produced by P. aeruginosa grown in continuous culture at D = 0.05 h^{-1} under nitrogen limitation on medium B, and (c) sodium alginate.*

aeruginosa, produces an acetylated alginate-type polysaccharide (7). In *Klebsiella aerogenes*, exopolysaccharide is produced from glucose under nitrogen, sulfate, and phosphate limitation but not under carbon limitation (18). This led to the suggestion (18) that, in this case, exopolysaccharide is the product of "overflow" metabolism. Other suggested functions of bacterial exopolysaccharides include protection against phagocytosis, desiccation and phage attack (19), involvement in uptake of metal ions (19), and participation in pathogen-plant interactions (17). In the normally nonmucoid *P. aeruginosa*, exopolysaccharide synthesis is known to be expressed only in connection with antibiotic (9) and phage (14) resistance and under certain pathological conditions (3, 14). The culture system developed here could be a useful tool in future studies of the physiological and clinical significance of exopolysaccharide synthesis by this organism.

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