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Isolation of a mutant strain of *Pseudomonas* sp. ATCC 31461 exhibiting elevated polysaccharide production

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A mutant strain of the bacterium *Pseudomonas* sp. ATCC 31461 that exhibited elevated production of the polysaccharide gellan on glucose or corn syrup as a carbon source was isolated. Gellan production by the mutant strain was about twofold higher than its parent strain on glucose or corn syrup after 48 h of growth, and about 1.4-fold higher after 72 h. An increase in biomass production was not correlated with enhanced gellan synthesis by the mutant strain. The increased gellan production by the mutant strain on either carbon source resulted in an increase in its culture medium viscosity and the viscosity of the isolated polysaccharide produced by glucose-grown cells. No differences in the glucuronic acid content of the polysaccharides produced by the mutant and parent strains were observed.
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Introduction

The polysaccharide gellan is produced by the bacterium *Pseudomonas* sp. ATCC 31461 [1,6,7]. This bacterial exopolysaccharide has a number of applications, including its use as a thickening agent or an agar substitute [8]. Gellan has a tetrasaccharide structure containing 20% glucuronic acid, 60% glucose and 20% rhamnose [5,11]. In the presence of monovalent and divalent cations, the gelation of this anionic heteropolysaccharide is enhanced [2,9]. *Pseudomonas* sp. ATCC 31461 utilizes glucose as a carbon source to synthesize gellan [1,7] as well as other carbon sources including corn syrup [3,12,17].

Despite a number of investigations exploring its structure or the physiological conditions that promote gellan production [2,5,11,16–19], few studies have attempted to isolate mutants of *Pseudomonas* sp. ATCC 31461 that exhibit enhanced polysaccharide production. A prior study has shown that the spontaneous variant strain, MP1, isolated from the rifampicin-resistant strain R40 [4], produced slightly higher gellan levels than its parent strain [10]. In this work, the isolation of a mutant strain of *Pseudomonas* sp. ATCC 31461 that exhibited enhanced polysaccharide production is reported. The isolated mutant strain was characterized partially and its properties were compared to those of its parent strain.

Materials and methods

Strains and media

The strains utilized in this investigation were *Pseudomonas* sp. ATCC 31461 [6,7] and strain EGP-1 that was isolated in this study. The strains were grown in a minimal medium that contained 0.05% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.09% soytone (hydrolyzed soybean meal) and 1 ml of a salt solution per liter of medium [19]. The salt solution contained 0.18% $MnCl_2 \cdot 4H_2O$, 0.248% $FeSO_4 \cdot 7H_2O$, 0.028% H_3BO_3, 0.003% $CuCl_2 \cdot 2H_2O$, 0.002% $ZnCl_2$, 0.007% $CoCl_2 \cdot 2H_2O$, 0.002% $NaMoO_4 \cdot 2H_2O$ and 0.21% sodium citrate$\cdot 2H_2O$ [9]. The carbon source glucose or corn syrup (3%) was added separately to the medium after autoclaving. The corn syrup utilized in this study is a dual acid enzyme conversion syrup that contains about 42% maltose. Batch cultures (50 ml) in 250-ml Erlenmeyer flasks were shaken (250 rpm) at 30°C over a period of 72 h after a nutrient broth ATCC 31461 culture grown for 48 h was used to inoculate each minimal medium culture to an initial concentration of approximately 10^6 cells/ml.

Mutant isolation

The mutant strain was isolated by a combination of conventional chemical mutagenesis and antibiotic resistance. Exponentially growing nutrient broth cultures of *Pseudomonas* sp. ATCC 31461 cells were treated with 1% (vol/vol) ethylmethane sulfonate (EMS) at 30°C for 60 min without shaking [15]. The cultures were diluted 1:30 in nutrient broth and shaken at 250 rpm overnight at 30°C [15]. The treatment of the bacterial cells with EMS resulted in 2.2% survival. Approximately 10^7 mutagenized cells were spread onto nutrient agar plates containing 100 mg/l ampicillin. After 72 h, colonies were visually inspected for their degree of polysaccharide formation. From a total of 2250 colonies, 23 putative mutants were screened for gellan production on 3% glucose with aeration (250 rpm) over a period of 48 h at 30°C. A mutant strain, designated EGP-1, was identified for its ability to elaborate higher gellan concentrations than ATCC 31461 after 48 h. Subsequently, gellan and biomass productions by strain EGP-1 and ATCC 31461 were compared over a period of 72 h.

Polysaccharide, biomass, viscosity and glucuronic acid determinations

A sample of culture medium (3 ml) was removed and placed in a boiling water bath for 15 min. The pH of the culture medium was

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Table 1 Polysaccharide production by *Pseudomonas* sp. strains ATCC 31461 and EGP-1 after growth on glucose or corn syrup as a carbon source

Fermentation time (h)	Polysaccharide (mg/ml) produced by strain relative to carbon source ±SD			
	ATCC 31461		EGP-1	
	Glucose	Corn syrup	Glucose	Corn syrup
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
24	1.96±0.10	1.04±0.14	2.27±0.27	2.17±0.06
48	1.98±0.20	1.98±0.10	4.49±0.64	4.83±0.12
72	2.60±0.29	2.24±0.25	3.62±0.10	3.20±0.00

Table 3 Culture medium viscosity of *Pseudomonas* sp. strains ATCC 31461 and EGP-1 relative to carbon source

Fermentation time (h)	Viscosity (cP) of culture medium ±SD			
	ATCC 31461		EGP-1	
	Glucose	Corn syrup	Glucose	Corn syrup
0	2±0	1±0	2±0	1±0
24	284±8	109±13	438±15	464±19
48	1658±36	1416±8	3087±78	3029±62
72	2935±90	2369±22	6846±88	4280±50

adjusted to pH 10 using 1 N KOH and then was incubated at 80°C for 10 min. The pH was adjusted to approximately 7.0 using trichloroacetic acid and was incubated at 25°C for at least 10 min. The medium was centrifuged at 20,400×*g* for 30 min at 25°C. The supernatant was used in the subsequent gellan determination while the cells were washed with water and centrifuged at 20,400×*g* for 30 min at 25°C. The washed cells were collected on preweighed 0.45-μm pore size filters (47 mm diameter) and dried at 80°C to constant weight. To quantitate the gellan concentration in the culture medium, 3 vol of ice-cold 95% ethanol were added to 1 vol of culture medium supernatant followed by vigorous mixing [20]. The mixture was maintained at −18°C for 20 min to enhance gellan precipitation. The precipitated polysaccharide was collected on preweighed 0.45-μm pore size filters (25 mm diameter) and dried to constant weight at 80°C. Viscosity of the culture medium (whole culture broth) or the polysaccharide in 3 mM potassium phosphate buffer (pH 7.0) was determined at 23°C using a Cannon LV2000 viscosimeter (State College, PA). The glucuronic acid content of each polysaccharide was determined at 410 nm using a previously described colorimetric method [14]. The Student's *t*-test was used during the statistical analysis.

Results and discussion

An attempt was undertaken to isolate a mutant strain of *Pseudomonas* sp. ATCC 31461 that exhibited enhanced polysaccharide production. Considering that gellan formation may share common nucleotide sugar pathway steps with peptidoglycan synthesis in this pseudomonad [10], a rationale was developed to screen ampicillin-resistant mutant strains for possible enhanced polysaccharide production. The mutant strain EGP-1 was isolated from its parent strain using a combination of chemical mutagenesis and its resistance to ampicillin. Strain EGP-1 was identified by virtue of its ability to synthesize higher gellan levels than ATCC 31461 after 48 h. Subsequently, a comparison of gellan production by strain EGP-1 and ATCC 31461 on glucose or corn syrup as a carbon source was made (Table 1). After 24 h, no significant difference in gellan production by the strains was observed when they were grown on glucose (Table 1). On corn syrup, a twofold difference ($P<0.01$) in gellan production by the strains was noted after 24 h (Table 1). Following 48 h of growth (Table 1), gellan elaboration by strain EGP-1 was more than double that observed for ATCC 31461 independent of carbon source, and the difference was significant ($P<0.01$). After 72 h of growth (Table 1), polysaccharide levels synthesized by the mutant strain were about 1.4-fold higher than the levels produced by the parent strain on either carbon source, which represented a significant difference ($P<0.01$) in gellan concentrations. It is not clear why the polysaccharide levels produced by the mutant strain after 72 h were lower than the levels observed after 48 h (Table 1). Perhaps, the EGP-1 polysaccharide after 72 h has become less ethanol-precipitable than the ATCC 31461 polysaccharide due to a structural change in the EGP-1 polysaccharide that does not affect its viscosity. In the closely related species *Xanthomonas campestris*, mutagenesis is also effective in the isolation of mutants capable of increased production of the polysaccharide xanthan [13]. The highest increase in xanthan production by the mutants isolated was about 1.4-fold after 72 h using glucose as a carbon source [13].

It was also of interest to learn whether the elevated gellan production observed for strain EGP-1 could be related to an increase in biomass production by the strain. Biomass production by strain ATCC 31461 was slightly higher than by strain EGP-1 after 24 h of growth on either carbon source (Table 2). The biomass levels produced by both strains were comparable after 48 or 72 h of growth independent of the carbon source utilized (Table 2). It was concluded that the elevated gellan production by strain EGP-1 on glucose or corn syrup was not due to an increase in its biomass production but may be the result of a mutation affecting

Table 2 Biomass production by *Pseudomonas* sp. strains ATCC 31461 and EGP-1 after growth on glucose or corn syrup as a carbon source

Fermentation time (h)	Cell weight (mg/ml) produced by strain relative to carbon source ±SD			
	ATCC 31461		EGP-1	
	Glucose	Corn syrup	Glucose	Corn syrup
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
24	0.93±0.00	1.35±0.04	0.79±0.08	1.03±0.03
48	0.90±0.07	0.92±0.10	1.06±0.10	1.13±0.06
72	1.13±0.06	1.14±0.14	1.37±0.20	0.88±0.00

Table 4 Viscosity of the polysaccharide produced by *Pseudomonas* sp. strains ATCC 31461 and EGP-1 relative to carbon source

Fermentation time (h)	Viscosity (cP) of polysaccharide ±SD produced by strain			
	ATCC 31461		EGP-1	
	Glucose	Corn syrup	Glucose	Corn syrup
48	244±12	279±17	419±8	214±4
72	208±11	366±4	669±26	410±25

Viscosity of each sample normalized to 1 mg/ml polysaccharide is given as cP where each result indicates the mean of three separate determinations ± SD.

Table 5 Glucuronic acid content of the polysaccharide produced after 72 h by *Pseudomonas* sp. strains ATCC 31461 and EGP-1 relative to carbon source

Strain	Glucuronic acid content of polysaccharide relative to carbon source[a]	
	Glucose	Corn syrup
ATCC 31461	0.27±0.03	0.18±0.02
EGP-1	0.35±0.08	0.21±0.02

[a]Content of the polysaccharide is expressed as micromoles of glucuronic acid per gram of gellan, where each result indicates the mean of three independent trials ±SD.

polysaccharide formation. In the previously isolated strain R40, the rate of biomass production appeared to parallel the rate of gellan production [10].

The viscosity of the culture medium for both strains was monitored over a period of 72 h. The glucose-based culture medium of strain EGP-1 was slightly more viscous than that of ATCC 31461 after 24 h (Table 3). In contrast, a fourfold difference was observed between strains EGP-1 and ATCC 31461 after 24 h of growth in the corn syrup-based culture medium (Table 3). After 48 or 72 h of growth of the glucose-grown cells, the viscosity of the culture medium of EGP-1 was 1.8- or 2.3-fold higher, respectively, than the viscosity of the culture medium of ATCC 31461 (Table 3). The highest culture medium viscosity observed in this study was determined for the mutant strain grown for 72 h in the glucose-containing medium (Table 3). For the corn syrup-grown cells, the viscosity of the culture medium of EGP-1 was 2.1- or 1.8-fold higher than the viscosity of the culture medium of ATCC 31461 after 48 or 72 h, respectively (Table 3). The differences in the viscosity of the culture medium for both strains after 24, 48 and 72 h of growth were significant ($P<0.01$), independent of carbon source. Similarly, the culture medium viscosity of strain R40 increased up to 100 h although gellan or biomass production failed to increase after 50 h [10]. It is not clear why the culture medium viscosities for the strains used in this study increased despite their polysaccharide and biomass levels remaining constant or decreasing. A possible explanation could be that significant water loss occurring within each culture between 48 and 72 h due to evaporation of water as the viscous medium is being shaken. Loss of water would raise the potassium and magnesium ion concentrations in the medium. The higher metal ion concentrations would likely increase the gel strengths of the polysaccharides [7] and account for the elevated polysaccharide viscosities observed after 72 h.

The viscosity of the polysaccharide produced by each strain was also compared (Table 4). The viscosities of the polysaccharide produced by ATCC 31461 after 48 h on glucose or corn syrup were about equal (Table 4). After 72 h, the polysaccharide produced by the corn syrup-grown ATCC 31461 cells was 1.8-fold higher than the polysaccharide produced by the glucose-grown ATCC 31461 cells (Table 4). The viscosity of the polysaccharide produced by glucose-grown EGP-1 cells was at least 1.6-fold higher after 48 or 72 h of growth than for the corn syrup-grown EGP-1 cells (Table 4). The viscosities of the polysaccharide produced by glucose-grown EGP-1 cells were 1.7- or 3.2-fold higher after 48 or 72 h, respectively, than those of its parent strain (Table 4). Differences in viscosity levels of the polysaccharide produced by the strains on glucose after 48 or 72 h were significant ($P<0.01$). This suggests that the polysaccharide produced by EGP-1 cells on glucose after 48 or 72 h has a higher molecular weight than the polysaccharide synthesized by its parent strain. The viscosity of the polysaccharide produced by corn syrup-grown ATCC 31461 after 48 h was higher than that of the polysaccharide elaborated by corn syrup-grown EGP-1 cells with a significant difference ($P<0.01$) in levels (Table 4). The difference in the viscosity levels of the polysaccharide elaborated by the strains on corn syrup after 72 h (Table 4) was not significant.

To further characterize the mutant strain, the glucuronic acid content of its polysaccharide produced after 72 h on glucose or corn syrup was compared with that of its parent strain (Table 5). The difference between the glucuronic acid content of the polysaccharide produced by strain ATCC 31461 or EGP-1 grown on glucose or corn syrup was not significant (Table 5). This analysis appears to indicate that the glucuronic acid content in the polysaccharide produced by the mutant strain was the same as that found in its parent strain. The glucuronic acid content of the polysaccharide produced by the spontaneous variant strain MP1 was also comparable to that of authentic gellan [10].

Overall, this study demonstrates that it is possible to isolate a mutant strain of *Pseudomonas* sp. ATCC 31461 that exhibits elevated gellan production on glucose or corn syrup as a carbon source. Elevated gellan production by the mutant strain did not appear to be caused by increased biomass production. Enhanced gellan production by the mutant strain is the likely explanation for the increased culture medium or polysaccharide viscosity relative to its parent strain. Lastly, the mutation in the mutant strain enhancing polysaccharide formation did not affect the glucuronic acid content of its polysaccharide.

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