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# Characterization of gellan-like polymers for new biotechnological applications

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**Abstract:** Studies on the mechanisms underlying the synthesis of bacterial exopolysaccharides (EPS) have raised great interest, being the non-pathogenic bacterium *Sphingomonas elodea* ATCC31461 one of the best examples. This bacterium produces gellan gum, traditionally used in food and pharmaceutical industries and more recently also in 3D-scaffold material for tissue engineering. To prepare scaffolds for chondrocyte proliferation, a polysaccharide that remains liquid slightly above body temperature, but jellifies at 37°C creating a 3D-structure is needed. Our aim was to search for gellan-like polymers fitting these requirements. To achieve that, mutations in the autokinase GelE of *S. elodea* were introduced and their effect on gellan properties determined. Mutations in the C-terminal  $\beta$ -strand and in Y198F led to lower viscosity polymers, while mutations N99A and Y209F led to EPS solution with higher viscosity. Native gellan and gellan-like polymers produced in the presence of GelE mutations N99A, C75K, V231E, and Y198F were further purified with organic solvents, being chloroform the best choice. Native gellan was the only one forming gels in the presence of Ca<sup>2+</sup>, but these gels were unstable in standard chondrocytes growth medium. In an attempt to find new bacterial polymers with potential applications, soil isolates of the genus *Massilia* were studied. These produced an extracellular polymer of unknown chemical composition, but with very different rheology from gellan. Overall, this study confirms the possibility to obtain gellan-like polymers by modifying proteins involved in EPS chain-length regulation. Since these polymers display different rheological properties, additional characterization should be made to find new potential applications.

**Key-words:** *Gellan gum; Gelation; Sphingomonas elodea; GelE autokinase; Rheological properties*

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## INTRODUCTION

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In the last years many studies have been developed to better understand the molecular mechanisms and regulatory processes underlying the synthesis of bacterial exopolysaccharides (EPS) as well as their potential applications. In the search solutions for biotechnological applications, gellan gum produced by *Sphingomonas elodea* ATCC 31461 emerges as a new promising extracellular polysaccharide due to its importance as commercial gelling agent. It is used in food and pharmaceutical industries and more recently in others fields, like biomedicine applications. The rheological proprieties of this hydrogel showed that it can be used in several

applications, such as construction of scaffolds in tissue engineering due to its ability as a cellular support or its multi-functional capacity like synergy with other polysaccharides, which allows the production of wide range of textures.

This commercial gelling agent, gellan, is an extracellular EPS produced by the non-pathogenic bacterium *Sphingomonas elodea* (*S. elodea*) ATCC 31461, formerly known as *Pseudomonas elodea* or *Sphingomonas paucimobilis*. It is aerobic, rod-shaped with a single flagellum and forms round mucoid yellow pigmented colonies in a defined medium (Kang and Veeder, 1981). This straight-chain is composed of a repeat-unit of four monosaccharide molecules, i.e., two D-glucose, one D-glucuronic acid and one L-rhamnose.

The native form of gellan also presents two lateral carboxyl groups on its glucose residue immediately after production: one L-glycerate and one of O-acetate (1 mol and 0.5 mol, respectively, per repeat-unit) (Omoto et al, 1999; Mota, 2010).

Figure 1 shows the typical fermentation process for gellan production and its purification at laboratory scale. *S. elodea* produces round mucoid, brilliant and yellow pigmented colonies (Figure 1, A1).

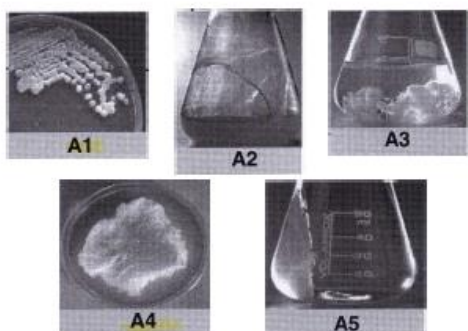


Figure 1 – Typical fermentation process for gellan production and its purifications at laboratory scale (adapted from Fialho et al, 2008).

The viscosity of the culture medium increases during the exponential and stationary phases and reaches, at the end of the process, a very high value (Figure 2, A2). Gellan production is growth-associated with a maximum production of 12 g/l. This increase of viscosity creates a great problem in terms of separating gellan from cells and before precipitation, it is necessary the dilution of the culture broth with saline solution in order to try reduce the viscosity, followed by centrifugation to separate de cells. The supernatant is precipitated with cold isopropyl alcohol (Figure 2, A3), dried and resuspended in water, followed by dialysis and lyophilization (Figure 2, A4). Finally gellan can be resuspended in water to produce a gel (Figure 2 A5), (Fialho et al, 2008).

Nowadays due to its rheological properties, gellan has been used in several application. US and EU approved this hydrogel as a gelling, stabilizing and suspending agent, either alone or in combination with other hydrocolloids in food and personal care applications (lotions, creams and toothpastes) (Fialho et al, 2008; Sá-Correia et al, 2002). It has also been employed as a gelling agent in plant biotechnology and in bacterial culture media in place of agar (Mota, 2010; Sutherland, 2002). Compared with other polysaccharides, gellan has many advantages such as an excellent thermal

and acid stability, adjustable gel elasticity and rigidity, high transparency and good flavor release (Fialho et al, 2008).

Gellan is commercially available in three forms according on acyl content. Without this group, Gelrite®, with low quantity, Kelcogel® F and with high values Kelcogel® LT100 (Mota, 2010). Icings and glaze sauces, microwavable foods, dessert gels, and puddings are food products that incorporate gellan.

More recently it was also shown that gellan can be used in ocular, nasal, gastric and drug delivery applications directly related with the biomedical field including its use as a pharmaceutical excipient (Fialho et al, 2008; Rozier and Mazuel, 1989; Deasy and Quigley, 1991). Hydrocolloid beads based on gellan have been shown to be useful for slow drug release (Gal and Nussinovitch, 2007). Li et al, (2001) showed that gellan might be an ideal candidate in the development of protein delivery systems, when a gellan film for insulin delivery in diabetic was implanted. Hydrogels based on natural and synthetic polymers have a special interest for cell encapsulation, drug delivery, serve as adhesives or barriers between tissue and material surfaces, and tissue engineering as matrix for repairing and for regenerating a wide variety of tissue and organs due unique biocompatibility, flexible methods of synthesis, range of constituents, and desirable physical characteristics (Hoffman, 2012).

So, in this work it was tested mutant polymers derived from gellan and verify if their properties could improve those of the gellan gum and consequently explore their ability to work as scaffold, drug delivery or encapsulating gel. To do that, the several gellan-like polymers were produced, quantified and viscosity measurement of a polymer solution was determined. For the selected ones, purification protocols were established and several tests were performed to assess gelation properties. It was also tested the ability of strains from the genus *Massilia* and their characteristics. The unknown polymer obtained from this strain was subjected to various tests, namely rheological tests with aim of understand in order to know if it could be used in biotechnological applications.

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## MATERIALS AND METHODS

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### Strains and plasmids, media and culture conditions

Strains used in this study were the native strain *S. elodea* ATCC 31461 that is the major producer of gellan; *S. elodea*  $\Delta$ *gelE* mutant strain (SpLM21-4) with the recombinant plasmid containing the *gelE* gene (pHA010-3) and other mutant plasmids with Y189F, D179N, V231E, C75K, G74A, Y121F, Y235F, Y209F, YF1F4, C75A, pBBR1MCS, pLM51-1, YF1F3F4, V227D, I228D, N99A, D79A, L232E. Strains from the genus *Massilia* were designated RAG-1 and RAG-2.

*S. elodea* strains were grown in S medium (Moreira et al, 2004) supplemented with the appropriated antibiotic. Strains RAG-1 and RAG-2 were grown in Lennox broth (LB) with or without of 5 g/l glucose (LBG). All strains were incubated at 30°C with 250 rpm if in liquid culture.

#### **Preparation of competent cells and electrotransformation**

*S. elodea*  $\Delta$ *gelE* mutant was inoculated into S medium and grown overnight at 30°C with stirring of 250 rpm. The pre-inoculum was added to 150 ml of S medium and grown until O.D. 600 nm of 1.2 was reached. After that, cultures were cooled on ice during 15 minutes and centrifuged during 15 minutes at 8000 rpm at 4°C. Supernatant was discarded and cell pellet was resuspend in 200 ml of deionized, cold and sterile H<sub>2</sub>O. This process was repeat four times, followed by different glycerol 10% volumes, 40 ml, 8 ml and 4 ml, respectively. The final cellular suspension was aliquoted in 100  $\mu$ l fractions and kept at -80°C. Prior to electrotransformation, plasmids were extracted using the *ZR Plasmid MiniPrep Kit* according to manufactures instructions. After that, cells and plasmid DNA were mixed and an electrical shock was applied. The cells were incubated with 1 ml of LB medium during 3 hours at 30°C with stirring and plated on to solid S medium with chloramphenicol. Plates were then incubated during 4 days at 30°C.

#### **Gellan production and purification**

The strains under study were inoculated in 250 ml of S medium and grown at 30 °C for 48 hours. After that time, the culture was diluted with NaCl to facilitate cell removal by centrifugation at 8000 rpm during 15 minutes at room temperature. The supernatant was precipitated with cold ethanol 96%, followed by successive washes with ethanol to remove cell debris. The polysaccharide was dried overnight and then weighted.

To further purify the polysaccharide it was necessary to test other approaches, such as treat the samples with chloroform, phenol and both. For that, 1g/l solutions of the different polymers were prepared in water. Then, to each 5 ml of sample were added 1.5 ml of ammonium acetate. Then, 5 ml of chloroform (or phenol) were added and the mixture centrifuged for 5 minutes at 13000 rpm. The aqueous phase containing the polysaccharide was recovered and stored at 4°C.

#### **Viscosity measurements**

The viscosity of each solution was determined at 30 °C using the viscometer Brookfield model DV-II with the spindle n. 40. Results are the means of at least three independent viscosity determinations.

#### **Methods for protein quantification**

The quantification of proteins was determined by two different approaches: the method Bradford and the method of Lowry.

#### **Gelation of modified gellan gum samples**

The selected samples of gellan-like polymers was mixed with sterile distilled water under constant stirring at room temperature. The solution was progressively heated at 90°C until homogeneous dispersion and maintained at that temperature for 15-20 minutes. Different concentrations of several salts, such as NaCl, CaCl<sub>2</sub>, and BaCl<sub>2</sub>.2H<sub>2</sub>O were added to the solution and progressively decreased the temperature until 40°C.

To test the gelation of the in Dulbecco's Modified Eagle's medium (DMEM) sample were kept in an incubator (37°C, 5% CO<sub>2</sub>).

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## **RESULTS**

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#### **Production and rheological properties of gellan-like polysaccharides**

*Sphingomonas elodea* produces gellan gum in high yield, giving rise to mucoid yellow colonies in plates containing S medium (Figure 2-A). Contrastingly, the deletion of the *gelE* gene encoding a putative tyrosine autokinase produces colonies that are slightly orange and dry (Figure 2-B).

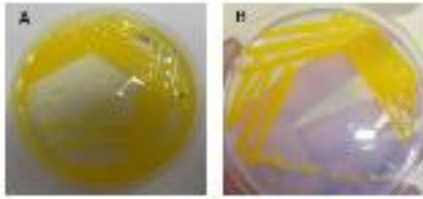


Figure 2 - Morphology of *S. elodea* ATCC (A) or  $\Delta$ gelE mutant (B) grown for 3 days at 30°C in solid S medium.

Analyses to GelE secondary structure evidenced several conserved regions, such as the ATP-binding pocket composed of the walker A, walker B and Mg<sup>2+</sup>-binding region, an amphipathic helice, a C-terminal  $\beta$ -strand, and several tyrosine residues (Figure 3).

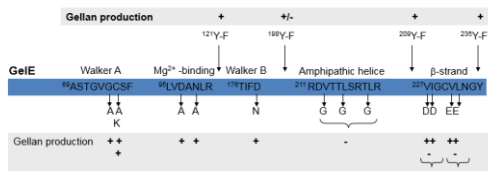


Figure 4 - Point mutations introduced into GelE coding sequencing and respective amino acid exchange. The effect of these mutations on gellan recovery from the growth culture is also indicated as + or -.

To evaluate the role of these regions in GelE activity regarding gellan production, several point mutations were previously introduced (Moreira et al, unpublished). The six mutations introduced in the ATP-binding pocket of GelE (G74A, C75A, C75K, D97A, N99A and D179N) still complement the gellan deficient phenotype of the  $\Delta$ gelE mutant (Figure 12). Contrastingly, the three simultaneous mutations in the amphipathic helice did not restore gellan production. When single mutations were introduced in the C-terminal  $\beta$ -strand domain (V227D, I228D, V231E and L232E) gellan was still produced, but the simultaneous deletion of two amino acids (VI or VL) prevent gellan production. As GelE is a putative tyrosine autokinase, 4 tyrosine residues were mutated (Y121F, Y198F, Y209F and Y235F). All of this mutations restored gellan biosynthesis by the complemented  $\Delta$ gelE mutant, although Y198F mutation gave rise to a lower amount of polymer and drastic changes in its rheology (Moreira et al, 2004). To understand whether these mutations were giving rise to gellan-like polymers with different rheological properties, viscosity of the growth medium and of a polymer solution, and quantity of produced polymers were determined. For that, the  $\Delta$ gelE mutant

was transformed by electroporation with the

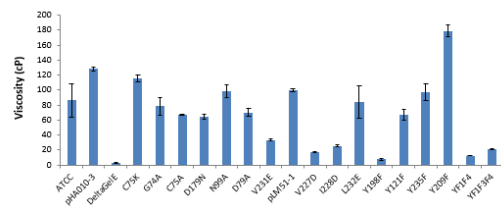


Figure 3 - Growth medium viscosity (shear rate 12 s<sup>-1</sup>) of *S. elodea* ATCC 31461 and  $\Delta$ gelE mutant complemented with the empty pBBR1MCS vector or with gelE carrying the indicated point mutations, after 48 hours of growth in S medium at 30°C with 250 rpm agitation. Data are average of three biological replicates.

empty vector (pBBR1MCS), with this vector carrying native *gelE* gene (pHA010-3), with pLM51-1 having the two last amino acids of GelE deleted, and plasmids carrying all the mutations of Figure 12 that still complement the EPS-producing phenotype. Three different colonies were chosen for each strain and grown in S liquid medium for 48 hours. After that period, growth medium viscosity was measured and data presented in Figure 4.

As expected, the growth medium of  $\Delta$ gelE mutant carrying pBBR1MCS does not show significant viscosity, but when complemented with *gelE* gene (pHA010-3), viscosity is restored to a level similar to the *S. elodea* wild type strain. Regarding the mutations in the different domains of GelE, the ones with higher impact in viscosity reduction were in the C-terminal  $\beta$ -strand (V227D, I228D and V231E) and in all containing the Y198F mutation ((Y198F; Y121F/Y235F (YF1F4) Y121F/Y209F/Y235F (YF1F3F4)) (Figure 4). This analysis showed that although all GelE variants restore gellan production by the  $\Delta$ gelE mutant, their effect on medium viscosity is variable, suggesting the present of polymers with different rheological properties.

To assess whether growth medium viscosity was comparable with the viscosity of the precipitated/dissolved polymer, the polysaccharide present in the culture medium of each strain was precipitated with ethanol, washed several times to remove cell debris and air dried. Then, 1 g/l solution of the gellan precipitates were prepared and viscosity was measured. Data here obtained confirmed previous results of Moreira et al (2004) since  $\Delta$ gelE supernatant precipitate does not show viscosity and the viscosity of the complemented mutant (pHA010-3) precipitated gellan does not reach the value of the wild-type *S. elodea* ATCC 31461

(Figure 5-A). In comparison to the viscosity of the polymer of the native GelE complemented strain, ATP-binding site mutation C75A, D179N, N99A and D79A have a positive effect in polymer viscosity (Figure 5-A). Mutations in the  $\beta$ -strand C-terminal region had different effects depending on the mutation. V231E, V227D and pLM51-1 had a negative effect on gellan viscosity while I228D and L232E led a position effect. Regarding the tyrosine residues, Y198F mutation had a negative effect, but Y209F mutation displayed a

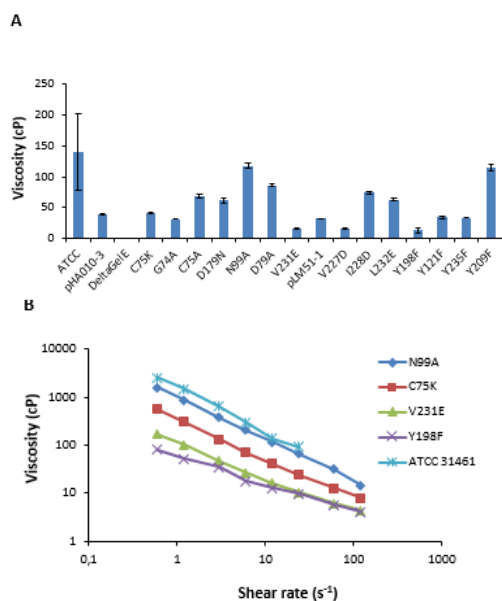


Figure 5 - Viscosity at 12 s<sup>-1</sup> (A) or at different shear rates (B), of the aqueous solutions prepared with 1 g/l of the ethanol-precipitated polymers isolated from the supernatants of the indicated strains, grown for 48h at 30°C in S medium. These bars (curves) are based on the median values of three viscosity measurements of polymer solutions obtained from three independent growth experiments.

strong increase of gellan solution viscosity (Figure 5-A).

The aim of the experiment shown in Figure 14-A was to identify strains producing polymers with different viscosities to assess properties such as gelation as will be further described. Therefore, we were choosing for further analysis strain N99A producing a polymer with a viscosity comparable to the wild-type ATCC 31461, C75K with polymer display 30% of the wild type and V231E and Y198F for lower polymer viscosity. Figure 14-B shows the viscosity at different shear rates for the above mentioned strains, confirming the different rheologies.

Next step was to assess if the strains producing the chosen gellans were good producers by determining the dry weight of the ethanol-precipitated polymer. Data

shown in Figure 6 demonstrate that V231E and C75K mutations give rise to 8.3 and 7.4 g/l, respectively, of polymer while Y198F produces approximately 4.4 g/l. The value for this last mutations confirms results previously published (Moreira et al, 2004). Due to unknown reason, no EPS was recovered from the strain with N99A

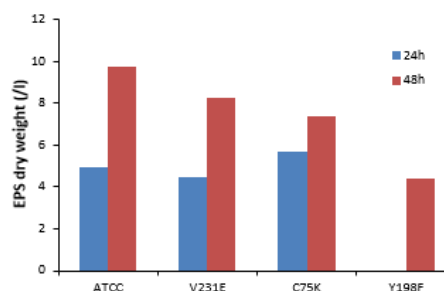


Figure 6 - Gellan production assessed by dry weight of the ethanol precipitated polysaccharide in the wild-type strain and when the mutant genes are expressed in the *S. elodea*  $\Delta$ gelE mutant.

mutation in this particular experiment and therefore no value can be shown.

### Purification and gelling properties of the gellan-like polymers

For future applications of these polymers, they need to be as pure as possible. Therefore, the next aim was to remove contaminants like proteins that collapse with the EPS when ethanol is added. As a first approach, EPS sample were run in a SDS-PAGE gel, but due to EPS viscosity, proteins were not separated (data not show). Then, purity of the EPS samples was followed by measuring the total protein content. For EPS sample deproteinization, samples were mixed with chloroform or phenol, centrifuged and the aqueous phase recovered. The protein content of the EPS solutions was determined by the modified Lowry method using the equation obtained in Figure 7.

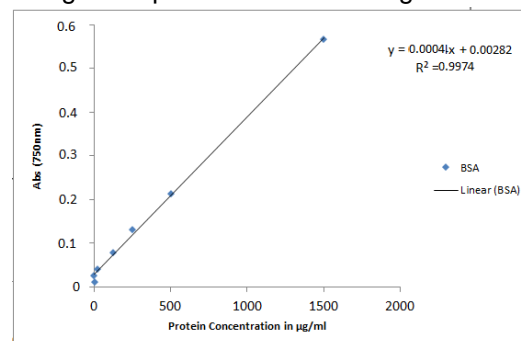


Figure 7 - Modified Lowry Protein Quantification Standard Curve prepared with serum bovine albumine (BSA).

Data obtained is shown in Table 4. Comparing with protein concentration of

untreated EPS samples, deproteinization using chloroform was effective for most of the samples, except for the native gellan. Nevertheless, significant amount of proteins were still measured for Y198F and N99A EPS. Samples treated with phenol were interfering with the Lowry method and negative concentrations or huge

Table 1 - Protein concentration in µg/ml of EPS samples.

Strain	Without treatment	Chloroform	Phenol
ATCC 31461	617.4	717.1	*
Y198F	1577.4	666.3	*
N99A	1036.6	674.6	*
V231E	522.2	71.5	7118.7
C75K	858.9	64.7	5407

\*negative values were obtained

concentration values were obtained (Table 1).

Due to interference of phenol with the Lowry method it was used instead the Bradford method to estimate protein concentration. After using BSA as standard for the calibration curve (Figure 10), the protein concentration are shown in Table 2.

Table 2 - Concentration in µg/ml of the proteins present in EPS samples by using the Bradford method.

Strain	Without treatment	Chloroform	Phenol
ATCC 31461	468.2	19.2	211.2
Y198F	497.7	275.2	591.2
N99A	438.2	245.2	269.2
V231E	778.2	239.2	739.2
C75K	959.7	223.2	456.2

The Bradford method seemed to be more suitable to determine EPS-samples protein contamination, since consistent values were obtained. According to this data the best compound for deproteinization of EPS samples was chloroform since it was observed, at least, a reduction of 50% in the amount of contaminant proteins (Table 2). After gellan-like polymers purification protocol was established, the following step was to determine the best conditions for gelation. It was known from previous published data that NaCl, CaCl<sub>2</sub> or sucrose could help in the gelation of gellan (Morris et al, 2012). But as sucrose could have detrimental effect or stem cell viability, only NaCl and CaCl<sub>2</sub> were tested. Using the native gellan produced by *S. elodea* ATCC 31461 and the  $\Delta$ gelE mutant expressing mutant GelE (V231E) in presence of NaCl, none of the samples made a gel (data not shown). When concentrations of CaCl<sub>2</sub> ranging from 0 to 200 mM were added to the EPS solutions, it was observed the formation of a gellified solution for the native polymer, especially to the concentrations around 20

mM CaCl<sub>2</sub>. An example of the type of gel formed is shown in Figure 8.

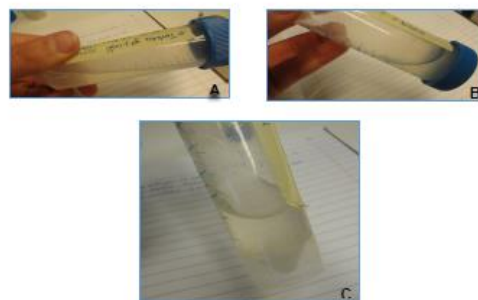


Figure 8 - Images showing attempts of gelation on V231E (A), C75K (B) and wild-type (C) polymers with CaCl<sub>2</sub>.

To determine the stability of the formed gel, drops formed with a syringe were transferred to water, PBS buffer and a 20 and 200 mM CaCl<sub>2</sub> solution. The gellified droplets were dissolved in water, but remained as a gel in PBS and CaCl<sub>2</sub> solutions. As the main goal of using gellan-like polymers would be to immobilize stem cells for growth and differentiation, it was need to test whether the gels produced in the presence of CaCl<sub>2</sub> were stable in DMEM medium, usually used to grown this type of cells. Therefore, a gellan solution of the native polymer was gellified with CaCl<sub>2</sub> (Figure 9-A) and then incubated overnight in DMEM with serum at 37°C. As shown in Figure 9-B, the gel must have dissolved because after removing the medium no gel-like structure remained.

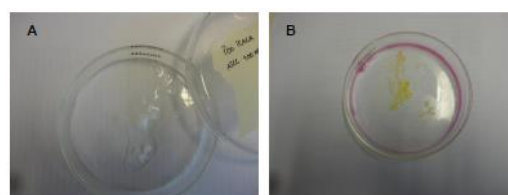


Figure 9 - Images showing the *S.elodea* ATCC 31461 native gellan after incubation with CaCl<sub>2</sub> and gelation (A) and after gel incubation in the presence of DMEM supplemented with serum overnight at 37°C (B).

Due to this last result, another ion (Ba<sup>2+</sup>) was tested for gelation. In spite of that, the gelation properties of gellan were not improved. This type of tests were also carried out with the gellan-like polymers V231E and C75K, but with these polymers no significant gelation was obtained (Figure 8).

#### Assessment of polymer production in strains of the *Massilia* genus

With the aim of finding new carbohydrate polymers with potential biotechnological applications, we started the characterization of two soil isolates, which after several days on LB plates, produce purple highly mucoid colonies. These two isolates, named RAG-1 and RAG-2, do not have a species attributed, but they may belong to the genus *Massilia* (Antônio Veríssimo, personal communication). The first experiment was to assess growth rate in two different media, LB and LB supplemented with glucose (LBG). Data shown in Figure 10-A indicate that the growth rate is similar for both strains in both media, but while RAG-1 maintained the same optical density in stationary phase, RAG-2 showed a slight decrease, suggestive of cell lysis. To quantify mucoidy, strains were cultivated in liquid medium, from which several samples were taken, and ethanol precipitated. Precipitated dry-weight increases along the growth period with RAG-1-LB having a maximum at 72 hours, RAG-1-LBG at 96 hours, and RAG-2 at 120 hours in both media (Figure 10-B). To evaluate if this polymer increased the viscosity of the growth media over time, we measured growth medium viscosity at different shear rates. Figure 10-C shows the results for the shear rate  $1.2 \text{ s}^{-1}$ . Despite some fluctuations, the general trend is an increase in viscosity, especially for strain RAG-1. Possibly due to the cell lysis observed in stationary phase, RAG-2 growth media shows lower viscosity when compared to RAG-1. As a final experiment to assess the rheology of these polymers, the ethanol-precipitable material produced by the two strains in both media were dissolved in water (1 g/l solution) and viscosity was measured. Data represented in Figure 10-D shows that the most viscous polymer at 92 hours of growth is from RAG-1 grown in LB. Although the medium viscosity for RAG-1-LBG at 96 hours is similar to RAG-1-LB, it shows considerably lower viscosity, implying that the RAG-1-LB polymer has higher molecular mass.

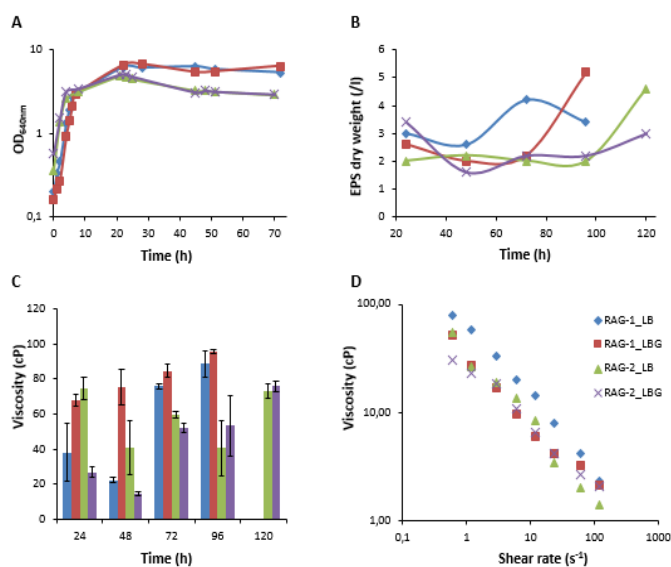


Figure 10 - (A) Comparison of growth curves of RAG-1 and RAG-2 grown at 30°C in LB or LBG media; (B) exopolysaccharide production, assessed based on the ethanol-precipitated supernatant; (C) growth medium viscosity at  $1.2 \text{ s}^{-1}$  shear rate; (D) viscosity at different shear rates of the aqueous solutions prepared with the same concentration (1g/l) of the ethanol-precipitated material isolated from different cultures grown, for 96 hours, at 30°C. These curves are based on the medium values from triplicate experiments. Strains legend for all the graphics is in (D).

## DISCUSSION

In this study it was evaluated whether different polymers produced by the  $\Delta gelE$  deletion mutant of *S. elodea* complemented with mutated variants of *gelE* gene could have different rheological properties suitable for cell tissue engineering approaches. GelE is expected to interact with membrane protein GelC (named activator domain) and regulate gellan chain length. Previous studies have shown that deletion of *gelC* or *gelE* gene leads to gellan production abrogation, but when a mutated variant of GelE having a phenylalanine residue instead of a tyrosine at position 198, gellan is still produced although in lower amount and with lower molecular mass (Moreira et al, 2004). This suggests that mutations putatively affecting the structure/function of GelE may result in different interaction with GelC and possibly to polymers with different rheological properties. To investigate this hypothesis, mutations of GelE conserved motifs were introduced by site-directed mutagenesis (Moreira, unpublished). In here, we analyzed the effect of these GelE mutations in the viscosity of the gellan-like polymers produced. Regarding the viscosity of the growth medium after 48 hours of

incubation, the mutations with higher impact in medium viscosity were in the C-terminal  $\beta$ -strand and Y198F which gave rise to lower viscosity and possibly to EPS with lower-molecular mass. Contrastingly, mutation affecting Y209F seems to indicate that an EPS with higher molecular mass is being produced. The C-terminal region of GelE seems to be determinant for gellan rheology, but the reason for this is unknown. An hypothesis is that this region is perhaps important for interaction with GelC, regulating then the activity of the polysaccharide polymerase enzyme GelG and consequently the size of gellan chains. When viscosity of the gellan-like polymers 1g/l solutions were determined, there was a good correlation with the growth medium viscosity. Exceptions are the mutations that lack the two last amino acids of GelE (pLM51-1) and I228D, with opposite effects. As representative polymers for further studies we were choosing mutation giving rise to polymers with high viscosity (N99A), intermediate (C75K) and low (V231E, Y198F). In addition we had determined the amount of EPS production in the presence of these mutations. With the exception Y198F mutation, all the others mutations do not interfere significantly with the amount of EPS produced. This is an important feature regarding possible biotechnological applications.

For putative application in tissue engineering, a gellan-like polymer needs to have the right rheological properties for cell growth and differentiation, but also requires a high degree of purity, not to be toxic to eukaryotic cells. Due to gellan viscosity it is not always possible to remove cells by centrifugation and quite often cells are precipitated with the polymer by the addition of ethanol. These samples are highly contaminated with bacterial proteins that have to be removed. In this work we tested whether organic solvents like phenol and chloroform, known for deproteinization could be used. Our data showed that chloroform was more effective in reducing the protein content of the EPS solutions, but still not all protein contaminants were removed. Phenol does not seem to be a good choice, because it is very difficult to remove from the EPS sample and is toxic to cells. Nevertheless, some extra work can be done for EPS sample deproteinization, such as incubation with a protease, followed by dialysis and lyophilization, or purification by chromatography.

Since an important goal was to test gelation of the different polymers, several conditions were tested. The aim was to obtain a polymer that is in the liquid state at temperatures higher than 37°C, but at body temperature makes a gel able to be used as scaffold for eukaryotic cell growth. With that in view we tested the native gellan in the presence of divalent cations such as  $\text{Ca}^{2+}$ , being able to produce a gel. When this gel was tested for stability in solutions containing salts (PBS,  $\text{CaCl}_2$ ) the gel was stable, but in the medium for stem cell growth, apparently the gel was dissolving. In addition, some of gellan-like polymers did not gelify under the tested conditions and other conditions have to be tested. One possibility is to crosslink these polymers with other compounds, to obtain a stable network of fibers with a tridimensional structure. Despite our results, this gellan-like polymers should not be left without consideration in further studies, because gellan is one of the few bacterial gums with gelling properties and one of the most promising tools for new biotechnological applications.

With the goal of searching for new bacterial exopolysaccharides with putative interest in the biotechnological field, we did the first preliminary studies with two soil isolates from the genus *Massilia* which comprises Gram-negative bacteria. RAG-1 and RAG-2 isolates produce a polymer which gives a mucoid morphotype to the purple colonies and that after cell-free supernatant precipitation gave a white material, not so dense as gellan. Viscosity measurements of the growth media or of a polymer solution showed very low values when compared to gellan, indicating that these polymers have different rheological properties. The nature of these polymers is unknown, and further studies including sugar content determination and structure determination should be conducted. From the two tested isolates, RAG-1 seems to be more promising since it has a highest production and viscosity when compared to RAG-2. Nevertheless, these are preliminary studies and new media, temperature of growth, pH should be tested. One important difference between *S. elodea* ATCC 31461 and RAG-1/ RAG-2 is that gellan production is stimulated by the high carbon/nitrogen ratio (S medium) while here we did not see significant differences between LB and LBG. A medium like the S medium was not suitable for growth of neither RAG-1 nor RAG-2 isolates.



Overall this work showed that mutations in proteins regulating polysaccharide chain length are good strategies to obtain polymers with different rheological properties. In addition, we started the characterization of other polymer producing microorganisms, hoping to find new interesting features for possible future applications.

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## REFERENCES

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Deasy, P., & Quigley, K. (1991). Rheological evaluation of deacetylated gellan gum (Gelrite) for pharmaceutical use. *Internacional Journal of Pharmaceutics*, *73*, 117-123.

Fialho, A., Moreira, L., Granja, A., Popescu, A., Hoffmann, K., & Sá-Correia, I. (2008). Occurrence, production and applications of gellan: current state and perspectives. *Applied Microbiology and Biotechnology*, *79*, 889-900.

Gal, A., & Nussinovitch, A. (2007). Hydrocolloid carriers with filler inclusion for diltiazem hydrochloride release. *Journal Pharmaceutical Sciences*, *96*, 168-178.

Hoffman, A. (2012). Hydrogels for Biomedical Applications. *Advanced Drug Delivery Reviews*, *64*, 18-23.

Kang, S., & Veeder, T. (1981). *Patent No. 4,377,636*.

Li, J., Kamath, K., & Dwivedi, C. (2001). Gellan film as an implant for insulin delivery. *Journal of Biomaterials Applications*, *15*, 321-343.

Moreira, L., Hoffmann, K., Albano, H., Becker, A., Niehaus, K., & Sá-Correia, I. (2004). The Gellan Gum Biosynthetic Genes *gelC* and *gelE* Encode Two Separate Polypeptides Homologous to the Activator and the Kinase Domains of Tyrosine Autokinases. *Journal of Molecular Microbiology and Biotechnology*, 43-57.

Morris, E., Nishinari, K., & Rinaudo, M. (2012). Gelation of gellan - A review. *Food Hydrocolloids*, *28*, 373-411.

Mota, B. (2010). Investigating the use of modified gellan polymers as scaffolding materials for cartilage tissue engineering. *MSc thesis in Biomedical Engineering*. Instituto Superior Técnico.

Omoto, T., Uno, Y., & Asai, I. (1999). The latest technologies for the application of gellan gum. *Progress in Colloid and Polymer Science*, *144*, 123-126.

Rozier, A., Mazuel, C., Grove, J., & Plazonnet, B. (1989). Gelrite: a novel, ion-activated, in-situ gelling polymer for ophthalmic vehicles. Effect on bioavailability of timolol. *International Journal of Pharmaceutics*, *57*, 163-168.

Sá-Correia, I., Fialho, A., Videira, P., Moreira, L., Marques, A., & Albano, H. (2002). Gellan gum biosynthesis in *Sphingomonas paucimobilis* ATCC 31461: genes, enzymes and exopolysaccharide production engineering. *Journal of Industrial Microbiology and Biotechnology*, *29*, 170-176.

Sutherland, I. (2002). A sticky business. Microbial polysaccharides: current products and future trends. *Microbiology Today*, *29*, 70-71.